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Respiratory Chain Activity and Protein Expression in Skeletal Muscles from High and Low Feed
Efficient Swine within the Same Genetic Line

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Food Science

by

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ABSTRACT

Current as well as past studies have provided evidence for a connection between macroeconomic/agricultural and cellular/biochemical basis of feed efficiency (FE; gain:feed) in farm animal production. Our investigation of respiratory chain activity, mitochondrial protein oxidation and protein expression was an expansion to the subcellular/molecular level. In this study we investigated the entire complex (Cx) of the electron transport chain (ETC) for differences in Cx activities in mitochondria isolated from skeletal muscle in high (HFE) and low (LFE) feed efficient swine within the same genetic line. Review of literature provided information for the connection between feed efficiency, mitochondrial function, and muscle type. Using UV spectrophotometry, gel electrophoresis and western blotting methods, we were able to study mitochondria from swine *Longissimus* (LM) and *Spinalis dorsi* muscles for differences in respiratory chain complex (I - V) activities, protein oxidation and protein expression of eleven subunits of the five electron transport chain complexes. We observed muscle dependent differences in Cx I and II, protein carbonyls and five subunits of the ETC (Cx I 30, Cor II, Cox IV, alpha and beta ATPase). We concluded after comparing our results with findings in literature, that respiratory chain activity protein oxidation and expression of protein subunits may be both skeletal muscle as well as a species dependent. These differential expressions may be the result of increased incidents of reactive oxygen species causing defects in Cx I or Cx II that may precipitate significant mitochondrial events and cellular pathways that provide links to the phenotypic expression of feed efficiency. Of high interest in relation to the mitochondrial link to FE are apoptotic, mTor and myostatin pathways.

ACKNOWLEDGEMENTS

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To my friends (Jim and Palika Dias Morse; Francisco and Morella Barretto Alvarez, who provided emotional and social support to my son, brother, and me; I am, forever, grateful to them.

DEDICATION

I dedicate this work to my family in appreciation for their belief in me. My Parents were adamant that I would always honor God, become a productive citizen, obtain a college degree, and remain humble even when completing requirements for the Doctor of Philosophy degree.

My family, James and Josephine Mead Alcorn (Parents), Andres Feliz (Husband) and Siblings: Dorothy, Lunia, Tillie, James, Jerry have passed away but continue with me in spirit as I walk the roads, climb the mountains and sometimes bottom the valleys of life only to rejoice with them at the finish line. Josephine, Freddy, Billy, and Terry are still with me and I am blessed for their being a part of my success and recipients of this dedication, with my son, J.R. Feliz and his two, Jeremiah and Jaeda, the joys of my life.

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ABBREVIATIONS

ATP (Adenine triphosphate)

ATPase α (a subunit of complex V with a molecular weight of 55.1 KDa)

ATPase β (a subunit of complex V with a molecular weight of 51.6 KDa)

ADG (Average daily gain)

ADFI (Average daily feed intake)

COX (cytochrome c oxidase)

Cox II or Cox2 (a subunit of complex IV with a molecular weight of 25.6)

Cox IV or Cox4 (a subunit of complex IV with a molecular weight of 19.6)

Cx (Complex)

Cx I (NADH coenzyme Q reductase)

Cx I 15 (a subunit of complex I with a molecular weight of 12.5 KDa)

Cx I 17 (a subunit of complex I with a molecular weight of 15.5 KDa)

Cx I 30 or C130 (a subunit of complex 1 with a molecular weight of 30.2 KDa)

Cor I (a subunit of complex III with a molecular weight of 51.6 KDa)

Cx II (Succinate-coenzyme Q reductase)

CX III (Reduced coenzyme Q-cytochrome c reductase)

Cx IV (cytochrome c oxidase)

Cx V (ATP synthase)

CYC c1 (cytochrome c1)

ETC (Electron transport chain)

FADH (flavoprotein adenine diphosphate)

FCR (Feed Conversion Ratio)

FE (Feed efficiency)

FeS (Iron sulfide---a subunit of Complex IV with a molecular weight of 29.6 KDa)

FIRE (Feed Intake Recording Equipment)

G:F (Kg gain:Kg feed intake)

GP (feed efficiency group P value)

HBP (Hexosamine biosynthesis pathway)

HFE (High Feed Efficient/efficiency)

Ip (Muscle group interaction P value)

Ip (Iron Sulphur Protein)

LFE (Low feed efficiency/efficient)

LM (Longissimus)

LRPPRC (Leucine Rich Pentatricopeptide Repeat Containing)—a protein coding gene

MP (Muscle group P value)

NADH (nicotinamide diphosphate)

Pi (inorganic phosphate)

Q (Ubiquinone)

RIP (Rieske iron sulfide protein)

ROS (Reactive oxygen species)

RNS (Reactive nitrogen species)

SpD (Spinalis dorsi)

TFI (Total feed intake)

TG (Total Gain)

UQPC (Ubiquinone binding protein)

UQCRQ2 (ubiquinol-cytochrome c reductase core protein II)

Wt (Body Weight)

RFI (Residual Feed Intake)

SCO (synthesis cytochrome c oxidase)

SURF-1 (SURFEIT 1, encodes for an assembly factor of Cx I, Cx IV)

UCP1 (uncoupling protein 1)

Chapter 1

INTRODUCTION

Feed represents 60-70% of the total cost in production of commercial livestock.

Therefore, feed efficiency (FE) remains one of the most important considerations in raising swine to market value. Lawrence and Fowler (2002) presented examples to define FE in terms of the recovery of energy in the body of an animal from the energy supplied in the food consumed by the animal. They listed four different ratios to describe feed efficiency:

- 1) Total energy in body gain/total energy in feed
- 2) Amount of feed consumed (kg unit time^{-1})/live wt. gain (kg unit time^{-1})
- 3) Gain of protein wt. in body tissues/wt. of protein provided in feed
- 4) Gain in muscle lysine/wt. of lysine in diet

They concluded that these examples provide a range for the possible relationships that demonstrate the macroeconomic and agricultural levels of efficiency that can be extended to cellular and biochemical levels. In our study FE was defined as total gain in body mass to total mass of feed intake (G:F) in kg unit time^{-1} . Since FE efficiency is such a complex entity and is not solely a function of carcass and feed intake weight, we considered growth rate an additional factor. In fact, total weight gain did not differ between FE groups in our study. We, therefore, assumed that growth rate was associated with compensatory growth, an increased growth rate succeeding a period of reduced feed intake, (Oksbjerg et al., 2017) in the HFE pigs.

Whittemore (1993) stated that growth rate is the controlling factor in the highly aggregate FE trait. Therefore, we equated fast and slow growth rate to high (HFE) and low (LFE) feed efficiency, respectively. According to Ruusunen and Puolanne (2004), some fast-growing pigs reach growth market value up to 25 days more quickly than slow growing pigs, and size of

skeletal muscles are important indicators of FE. They reiterated that pigs are generally selected for their ability to grow rapidly, that intensive selection for fast growth has precipitated a change in favor of light over dark muscle fibers and that the most economically important muscle is the *Longissimus* (LM). A mechanism for such change may involve the mitochondria since light and dark muscles are, respectively, abundant in glycolytic and oxidative type fibers (Morita et al., 1970). Light muscles are low in mitochondria number and capillary density compared with dark muscles and are also dominant in fast growing pigs, according to Ruusunen and Puolanne (2004). Additionally, Oksbjerg et al. (2017), reported that compensatory growth, not normal growth, is an important factor affecting feed efficiency. Additionally, Gilbert et al., (2017), in a review of divergent selection for residual feed intake (RFI), reported findings for increased lightness of meat in pigs with low RFI. The changes were said to be associated with muscular metabolism. Therefore, it appears that the phenotypic expression of FE may be linked to both muscle type and mitochondrial function.

The *Longissimus* (LM) and *Spinalis dorsi* (SpD) are separate representations of light and dark muscle fibers, respectively and are also parts or branches of one larger muscle. Since LM and SpD represent light and dark muscle, with low and high mitochondria density, respectively, we examined respiratory chain activity, protein oxidation, and specific protein expression in the two muscles separately. On the other hand, LM, SpD, and *Illiocostalis* make up one larger muscle, identified as the *Sarcospinalis* in swine. Therefore, combined values were also measured, in search of interactive indicators for the phenotypic expression of feed efficiency.

The mitochondrial respiratory complexes include Cx I, (NADH-ubiquinone oxidoreductase), Cx II (succinate ubiquinone oxidoreductase), Cx III (ubiquinone cytochrome *c* oxidoreductase), Cx IV (cytochrome *c* reductase), and Cx V (ATP synthase). A total of eleven

proteins from the respiratory chain complexes were also evaluated for expression differentials. The respiratory chain complexes are responsible for the transfer of electrons from metabolic substrates to the final electron acceptor, oxygen. The energy produced in the process of transferring electrons from NADH or FADH to Cx I and Cx II, respectively, to Cx III and then to Cx IV) is made available for the synthesis of ATP via ATP synthase (Cx V).

ATP synthesis occurs in the inner membrane of the electron transport chain (ETC) and includes the production of reactive oxygen species (ROS), which may be responsible for compromising the integrity of the mitochondrion. Destabilization of the mitochondrion may result in not only electron leakage but also movement of cytochrome *c* to the cytoplasm. In the event of cytochrome *c* leakage, mitochondrial apoptosis could occur, whereby myonuclear decay would result in a decrease in abundance of mitochondria. Since red muscle is comprised of an abundance of mitochondria compared to white muscle, red muscle is more receptive to ROS production and subsequently myonuclear decay. An overabundance of these events may be reflected in mitochondrial inefficiencies. According to Ruusunen and Puolanne (2004), the selection process has resulted in an increase in light over dark muscle. Bottje et al. (2002), however, linked FE to mitochondria function. Thus, in our study a combination of Ruusunen/Puolanne's muscle type and mitochondrial links to FE (Bottje et al., 2000) were taken into consideration as we searched for a marker or mechanism to elucidate the phenotypic expression of FE in swine.

In their study in 2002, Bottje et al. reported that mitochondria isolated from breast meat of HFE chickens had higher complex (Cx) activities than mitochondria from LFE birds. Lutz and Stahly (2003) presented similar findings in rats. Iqbal et al. validated these findings in 2004, suggesting that the differential expression of specific mitochondrial proteins may be a

compensatory response to lower complex activities of the electron transport chain (ETC). These findings may link phenotypic expression of FE to muscle type and mitochondrial function. Such a link could support the involvement of a process precipitated by reactive oxygen species (ROS)-mediated defect in Cx I (Saada et al., 2004) or Cx II (Senoo-Matsuda et al., 2003).

The mitochondrion houses the ETC (Figure 1-1) and is thereby responsible to produce ATP (Senoo-Matsuda et al., 2003), ROS (Zhao et al., 2004), and apoptosis (Li et al., 2003). ATP synthesis, ROS production and apoptosis are associated with complex activities, oxidative degradation in muscles, and mitochondrial proteins (Adhihetty and Hood, 2003). Since red muscle is more susceptible to ROS production, Loureiro et al. (2016) and HFE pigs fed a high lean diet have been associated with a higher degree of glycolytic (Vincent et al., 2015) metabolism, we predicted higher respiratory chain activities and protein expression in HFE mitochondria and the LM compared to LFE mitochondria and SpD. However, Fu et al. (2017), in a comprehensive proteome of HFE and LFE porcine skeletal muscle tissue, reported an inverse relationship between oxidative phosphorylation/differentially expressed mitochondria-located proteins and FE. The conclusion was that mitochondrial energy metabolism in porcine skeletal muscle is negatively correlated with FE.

The purpose of this research was to determine if a relationship between complex activities and protein expressions from skeletal muscle mitochondria in swine and phenotypic expression of FE could be established, as has been reported in broiler breast and leg muscles (Bottje et al., 2002). Ultimately, we were searching for a marker or mechanism that may support a molecular pathway for the phenotypic expression of FE in swine by measuring respiratory chain activities, and protein expression in porcine LM and SpD samples.

Specific objectives were

- 1) To measure respiratory chain complex activities (Cx I to Cx V) in mitochondria isolated from LM and SpD muscles from HFE and LFE swine within the same genetic line
- 2) To determine if mitochondrial proteins and degree of protein oxidation differ in HFE and LFE swine skeletal muscles from pigs within the same genetic line
- 3) To evaluate the difference in expressions of selected mitochondrial proteins from LM and SpD in HFE and LFE swine within the same genetic line

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Figure

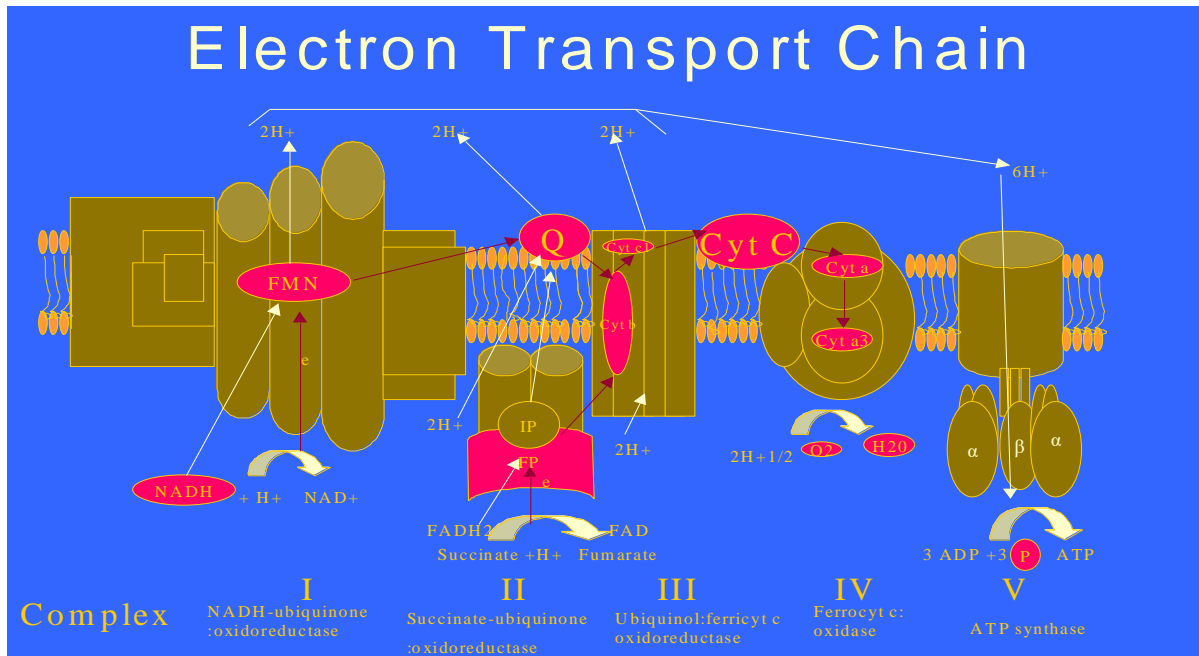


Figure 1-1. Electron Transport Chain. The transfer of electrons from the NADH and FADH₂, down the chain to the final acceptor, oxygen, is represented by red arrows, whereas the white arrows show the flow of protons from the matrix to the intermembrane space.

Moghadam et al., 2012

Chapter 2

REVIEW OF LITERATURE

Introduction

Feed represents 50 to 70 % of the total cost for raising livestock to market value.

Therefore, feed efficiency (FE) remains the most important factor associated with commercial farm animal production. According to Whittemore (1993), historically, genetic selection for improved feed conversion at the farm level can be attributed to increase in growth potential and the associated selection for lean. He reiterated that an animal growing slowly will incur the same feed maintenance costs, without the increase in product to offset the nutrient cost of the feed. Hence, rapid growth remains the most important controller of feed conversion. Whittemore (1993) also stated that the ratio of lean:fat in growth is second only to growth rate as a prime determinant of FE. He then reiterated that feed energy cost of fatty tissue growth is approximately four times that of lean tissue growth, due to differing water content of these two tissues. Therefore, in addition to procuring information on FE, respiratory chain activities, and protein expression in skeletal muscle, the link between FE and growth rate was also of major interest in this review.

Feed Efficiency

Feed efficiency (FE) is defined in ratio format to equal output/input or input/output by Lawrence and Fowler (2002), who gave as example the recovery of energy in the body of an animal from the energy supplied in the food consumed by the animal. They listed four different ratios to describe feed efficiency:

- 1) Total energy in body gain/Total energy in feed

- 2) Amount of feed consumed (kg unit time⁻¹) /Live wt. gain (kg unit time⁻¹)
- 3) Gain in wt. of protein in body tissues/wt. of protein provided in feed
- 4) Gain in muscle lysine/wt. of lysine in diet

They concluded that these examples provide a range for the possible relationships that demonstrate how the macroeconomic and agricultural levels of efficiency can be extended to not only cellular and biochemical levels, but also to the subcellular and molecular levels.

Observations from the study of mitochondrial function, respiratory chain complex activities, involving the transport of electrons, and mitochondrial protein expression in swine skeletal muscles could provide clues to elucidate the mechanism for phenotypic expression of feed efficiency.

Whittemore (1993) defined FE as a measure of nutrient utilization and feed conversion, which is a complex highly aggregate trait and net result of the interaction between different component traits. He added that underlying factors include basal metabolism, protein accretion, production level, livability, appetite, digestibility, carcass composition, behavior, and growth rate, the prime determinant of feed conversion. Alvarez (2011) reiterated the diversity in factors impacting FE. He investigated the compensatory growth response (CGR). The CGR is a rapid growth phase following a period of decreased feed intake (FI), which, according to Heyer and Leybret (2007), affects growth performance, composition of weight gain, at carcass and muscle levels. With this information, we continue with the premise that growth rate is the controlling factor in FE, but is impacted by various traits, an especially important one being FI. Pitchford (2005) added that heritability is a factor in FE as well, with the mean heritability in FE from 35 estimates across 7 species being 0.25 ± 0.02 . According to Solanes et al. (2004), high growth rate has been associated with HFE and is such an important economic trait in pig production, that

it is included in all breeding evaluations with phenotypic post-weaning growth measurements from approximately 25 kg to market weight. Pritchard (2005) stated that increased net FE correlated to increased fatness, but in pigs and beef cattle there has been some evidence of the reverse. He reported that poultry and mouse studies reveal an association of increased net FE with decreased heat production.

The trend in carcass consideration in HFE animals is in the direction of growth of lean skeletal muscle and decreased back fat with an increase in light compared to dark muscle (Ruusunen and Puolanne, 2004). Lorenzen et al. (2000) reported that Callipyge lambs with enhanced muscle growth, expressed a reduction in protein degradation rather than an increase in protein synthesis and that the muscle growth is due to hypertrophy. Therefore, number of oxidative and glycolytic skeletal muscle fibers is established in the process of myogenesis (Banda et al., 2004) and could be connected to respiratory chain activity. Because oxidative fibers have significantly higher numbers of mitochondria than glycolytic fibers, oxygen consumption is greater in oxidative compared with glycolytic fibers. Hence, the production of ROS would be expected to occur more often in SpD than LM. Reactive oxygen species in excess could destabilize mitochondria and lower electron transport chain efficiency. Therefore, the process of myogenesis is distantly related to oxidative phosphorylation and respiratory chain activity, since the number of mitochondria, established prior to birth, determines the degree of oxidative phosphorylation.

Mitochondria and FE or Residual Feed Intake (RFI)

According to Grubbs et al. (2012), the importance of mitochondria in feed efficiency is apparent, as they account for 90 % of the ATP and use the major portion of energy produced in

the body. The efficiency can be measured by Feed Conversion Ratio (FCR) or RFI (Jing et al., 2015), which is the difference between the observed intake and that predicted from the average requirements for growth and maintenance (Cai et al., 2008). Grubbs et al. (2012), in a study with pig production, found a positive correlation between RFI and ROS from LM mitochondria and concluded a selection for low RFI significantly decreased feed requirements. In support of these findings, we use low RFI and high RFI in referring to HFE and LFE, respectively.

Bottje et al. (2006) in an investigation between feed efficiency and mitochondrial function, reiterated the possibility of the mitochondrial contribution to lower electron transport activity in male broilers. They reported that greater uncoupling of the ETC in LFE mitochondria was correlated with specific defects in the electron chain and higher amounts of ROS. Higher mitochondrial ROS in several tissues in LFE broilers were likely responsible for higher protein carbonyl levels, indicating greater protein oxygenation and damage (Bottje et al., 2006). Accordingly, higher protein damage in poultry mitochondria may have been a factor in respiratory chain activity. Also, feed intake plays a major role in mitochondrial function, as Sharifabadi et al. (2012) reported that, in LM muscle in fat tailed lambs, respiratory chain activity in all 5 complexes, the low residual feed intake (RFI) mitochondria exhibited higher electron transport activity compared with high RFI. Using FCR, only complex activities III, IV, and V were less in low FCR. The low FCR animals gained 70 g more per day compared to high FCR. Zamir et al. (2015) studied pre- and post- slaughter respiratory activity in Ghezel muscle from random bred lambs. The results were not significantly different between the two groups in complexes I, III, and V; however, complex II and IV activities were greater in the post-slaughtered group. Hepatic mitochondria from Hereford steers were investigated for mitochondrial function in relation to complex activities, density, and protein expression (Casal,

et al., 2018). Their investigation showed greater mitochondria density, mRNA, DNA, and citrate synthase in low compared to high feed efficient Hereford Steers.

Skeletal Muscle

Process of Muscle Development

Feed efficiency in farm animals and especially swine is intimately connected to muscle development. Lassiter et al. (2019) found that high FE muscle development in Pedigree Male broilers, the down regulation of the myostatin signaling pathway coupled with the upregulation of formation and growth genes. Therefore, the process by which muscle develops is of crucial importance as the energy requirement affects the input/output ratio. Two important processes for muscle development exists.

Myogenesis is the prenatal process by which number of muscle fibers is established. Skeletal muscles are classified as either red (slow twitch- type I) or white (fast twitch- type II) depending on their speed of reaction to stimulation (Banda et al., 2004). Therefore, energy requirements between the two types of muscle differ. The process of myogenesis includes precursor cells, proliferation, fusion to form myotubes and finally differentiation into myofibers. Differentiation and growth are both attained by protein accretion and cell proliferation (Wigmore and Evans, 2002). Picard et al. (2002) made a point of emphasizing that number of fibers in skeletal muscle is fixed during myogenesis in fetal life or soon after birth of farm animals (pig, poultry, and rabbit) but reiterated that some plasticity occurs later in all species.

Postnatal growth proceeds via hypertrophy rather than hyperplasia. The process is accompanied by proliferative activity of satellite cells, which provide the new nuclei that are incorporated into muscle fibers. Most authors report that muscle fiber number remains

unchanged after birth. In the fish, however, the number of muscle fibers increases throughout life, but hypertrophy becomes increasingly important to growth (Strickland, 1983). Muscle fibers grow towards a plateau, whereas fiber number remains almost constant (Fiedler, 1983; Rehfeldt et al., 1987; and Wegner et al., 2000).

Classification of skeletal muscle and the mitochondrial link to feed efficiency

The basic classification of skeletal muscle fibers into glycolytic and oxidative makes them prime candidates for studies involving the transport chain system due to number of mitochondria associated with the different muscle types. Earlier in this review, HFE and LFE were equated to fast and slow growth as FE was defined. High feed efficient (HFE) pigs have the propensity for more light muscle fibers and the *Longissimus* (LM) is one of, if not the most economically important muscle in pigs (Fielder et al., 2014; Ruusunen et al., 2004).

Mitochondrial function and the mitochondrial link to FE were pioneered by Bottje et al. in 2002. Iqbal et al. (2004) continued the investigation of the mitochondria and validated the connection to FE. Fielder et al. (2004) and Ruusunen and Puolanne (2004) provided a link to fiber type. Information on the fiber type link was of interest because our study with porcine LM (white or light) and SpD (red) muscles represent two different muscle types, glycolytic and oxidative, respectively. Muscle types differ on the basis kind and abundance of specific myofibers.

Myofibers can be categorized as type I (slow-twitch), IIA (fast-twitch) and IIB (fast-twitch) or metabolically as oxidative, oxido-glycolytic, and glycolytic, respectively (Larzul et al., 1997). Types I and IIA have a high concentration of mitochondria and capillary density, whereas type IIB fibers are comprised of low mitochondria numbers and low capillary density (Ruusunen and Puolanne, 2004). Type I (red; slow twitch is the smallest, with a highly oxidative character and higher fatigue resistance compared to the IIB (Hamalainen and Pette, 1993), which

has high mitochondria volume. Leary et al. (2003) stated that mitochondria in vertebrate striated muscles can range from < 1% of cell volume to > 45% of cell volume. They stated that the content of mitochondria in muscle reflects the relative importance of mitochondria to the energy budget of each fiber type.

Karlsson et al. (1999) reported that muscles differ in fiber composition, rate of fiber differentiation and rate of muscle growth. They concluded that most muscles have a fiber composition, which is a mix between light and dark fibers. Dark muscles such as the SpD contain primarily oxidative type IA and IIA muscle fibers, while light muscle such as the LM has a high degree of glycolytic type IIB fibers (Ruusunen and Puolanne, 2004). The fibre type composition may be an important indicator of the mitochondria involvement in phenotypic expression of FE, since they suggested that intensive selection for fast growth in swine has precipitated a change in favor of light over dark muscle fibers. If growth rate is indeed the controlling factor in feed efficiency as suggested by Whittemore (1993), then high feed efficiency (HFE) may be equated to fast growth. Additionally, Leary et al. (2003) revealed that mitochondria volume in vertebrate striated muscles can range from < 1% of cell volume to > 45% and oxidative type fibers and have a high concentration of mitochondria, whereas the mitochondria number is low in the glycolytic type fibers (Moyes and Hood, 2003). Type I and IIA fibers are normally smaller and are surrounded by more capillaries than type IIB fibers (Kallstrom, 1995). Thus, oxygen consumption is higher in type I as compared to type II fibers, with an increased incidence of ROS production and subsequently greater chance for mitochondrial destabilization in type II and type IIA compared with type I fibers.

Longissimus and Spinalis dorsi

Longissimus (LM) and *Spinalis dorsi* (SpD) represent light and dark as well as glycolytic and oxidative muscle types, respectively. The differences in the muscle types are related to the number of mitochondria, high in dark and low in light muscles. Therefore, information gathered from studying respiratory chain activity and protein expression in LM and SpD may provide indicators for phenotypic expression of FE. These muscles are included in the category of deep muscles of the back according to Gray (2000) and of the trunk, neck and head or extensors and include the loin (Frandsen and Spurgeon, 1992). They are two of three columns forming the *Sarcospinalis*; *Iliocostalis* is the most lateral, *Longissimus* an intermediate and *Spinalis* the medial (Gray, 2000). The LM is largest of the loin muscles and its growth in swine is an indicator of FE, making it the most economically (Ruusunen and Puolanne, 2004) important among the various skeletal muscles in swine.

Since phenotypic expression of FE may be associated with the propensity for increased growth of light over dark muscles, increase in growth of LM may occur at the expense of SpD and *Iliocostalis*. As ATP provides the necessary energy for cellular processes and its availability depends on electron transport chain as a function of respiratory chain complexes, a study including respiratory chain activities and associated protein subunits may provide insight into the molecular level of FE. Feed efficiency studies with white or light skeletal muscles usually include the simultaneous study of dark muscles.

SpD is rich in type I (oxidative, aerobic slow-twitch) and LM type II (glycolytic, anaerobic fast-twitch) fibers, hence more oxidative damage by reactive oxygen species may be expected in LM compared to the damage in SpD because of the lower concentration of enzyme antioxidants in type II compared to type I fibers (Koo-Ng et al., 2000). In their study with rats,

they observed that after a six-week exposure to hydroxyl or superoxide radicals, a type II rich muscle (plantaris) was higher in carbonyl than a type- I- rich muscle (soleus) without protein damage. They hypothesized that type I muscles have a greater capacity to protect against damage from reactive oxygen species (ROS) as compared to type II muscles. However, in the same study, the capacity of type I muscles to resist hydroxyl radical protein damage was significantly reduced after six-week exposure to ROS. Hence, continued exposure to ROS could change the dynamics of the muscle type in its capacity to resist damage. Just as the continued exposure to ROS lowered the ability of the red type I muscle (soleus) to resist protein damage, a trade-off to high feed efficiency may be enhanced ROS production, which could be significant in characterizing the phenotypic expression of feed efficiency. The role muscle or fiber type plays in FE could be tested by measuring respiratory chain activities and protein expression of transport chain subunits, since muscle types are classified according to their glycolytic (type II) and oxidative (type I) capacities, based on mitochondria abundance.

Mitochondria, Respiratory Chain Activity, and Protein Expression

Introduction

Mitochondria are the bioenergetic and metabolic centers of eukaryotic cells. Their gross morphology is similar in all tissues, according to Tzagaloff (1982), and the structure is important for their role in maintaining homeostasis in epithelial tissue (Vlasta et al., 2003). The fact that intestinal epithelium lays close to a dense microbial milieu, renewed every 3-5 days, where pluripotent stem cells reside at the crypt, differentiate, and migrate to the tip of the villus is key to establishing a metabolic check point for equilibrium (Rath et al., 2018). They stated that during these transitions, changes in mitochondrial activity reflected intestinal epithelial cells

(IECs) distinct metabolic identities as key players in coordinating cellular metabolism, immunity, stress response and apoptosis, thereby, representing the source of the gate-keeper's function of the mitochondria in this tissue.

The mitochondrion is also a central architect of copper homeostasis and copper is required for the survival and development of all eukaryotes (McCarron et al., 2013). Some of the findings in 2013 were the following: mitochondria consist of two membranes, separating the organelle into four distinct compartments. The outer membrane contains porins, which permit free diffusion between the outer and inner membranes. The intermembrane space contains proteins such as cytochrome *c* that play major roles in mitochondrial energetics and apoptosis. The inner membrane is highly impermeable to most ions and molecules, which require transporters to cross. It contains 20% of the total mitochondrial protein, e.g. translocase and enzymes of the ETC. The matrix contains the majority of enzymes responsible for the citric acid cycle reactions. In contrast to Tzagaloff (1982), McCarron et al. (2013) painted a picture of diversity in the mitochondria, citing findings of structural differences per the tissue.

The electron transport chain (ETC, Figure 2-1) site of oxidative phosphorylation is located within the mitochondrial inner membrane. Senoo-Matsuda et al. (2003) explained that the ETC is intimately responsible for three important processes: 1) ATP production, 2) generation of ROS and 3) apoptosis. These processes in concert may involve the mitochondria in a pathway leading to the elucidation of a mechanism for the phenotypic expression of FE in swine. Compromised structure of the mitochondria can negatively impact FE in that energy can be more readily lost and manifested via activities of the five complexes (Cx I to Cx V) of the ETC. Knowledge of the origin and makeup of the five complexes is useful to understand how respiratory chain activity can relate to FE. Kumari (2018) gave his rendition of the electron

transport chain as he described the complexes as part of the mitochondrial pathway where electrons travel through a redox span of 1.1 volts. He explained electron transport from NAD⁺/NADH to the terminal electron acceptor.

For example:

CoQ

NADPH Linked: Cx I → → → Cx III → Cx IV → V (ATPase).

CoQ

Succinate Linked: Cx II → → → Cx III → Cx IV → Cx V (ATPase)

Complex I (Cx I)—NADH ubiquinone:oxidoreductase is the largest of the five ETC complexes with 30 nuclear encoded subunits, while 7 are mitochondrial DNA encoded. Cx I is in the inner membrane and mitochondria matrix and associated with complexes III and IV via Respirasome as super complex (Sousa et al., 2016). Nuclear mutations in Cx I are more common than mitochondria mutations. The function of Cx I is the transfer of electrons from NADH to ubiquinone. With the cofactor flavin mononucleotide, the transport takes place via the redox groups, iron-sulfur clusters (Andrews et al., 2013).

Complex II (Cx II)—succinate:ubiquinone oxidoreductase consists of only 4 subunits, all of which are nuclear encoded. They are the flavoprotein SDHA (Fp), Iron-sulfur protein SDHB (Ip), and two cytochrome *b* subunits (SDHC and SDHD, which bind Cx II to the matrix side of the inner membrane (Figuerola et al., 2002).

Complex III (Cx III)—ubiquinol: ferricytochrome c oxidoreductase was described by Barrel et al. (2008) as comprising ten nuclear and one mitochondrial subunit. The nuclear subunits include cytochrome *c*1 (CYC1), ubiquinone binding protein (UQPC), ubiquinol-cytochrome *c* reductase core protein II (UQCRC2) and Rieske FeS protein (R Ip). The one

mitochondrial subunit is the cytochrome *b* (cyt *b*). Cx III is in the inner mitochondrial membrane and is associated with super complexes of Complex III and IV via cardiolipin. Cx III functions to transfer electrons from ubiquinol to cytochrome *c* (cyt *c*) via the three redox centers (cyt *b*, cyt *c*, R Ip). No nuclear mutations have been identified with Cx III. Mitochondrial mutations are associated with cyt *b*.

Complex IV (Cx IV)—ferrocytochrome c:oxidoreductase as described by Coenen et al. (2006) is a 13-subunit complex, comprising 10 nuclear DNA and 3 mitochondrial encoded subunits. The nuclear subunits have structural and regulatory roles, whereas the larger mitochondrial COX subunits serve as a catalytic core, with three copper and two heme molecules as prosthetic groups in a holoenzyme for direct involvement in electron transfer. Assembly factors include components of biosynthetic pathway, individual COX subunits, and prosthetic groups to apoenzyme (for example, Surfeit-1 (SURF-1), SCO1, SCO2, COX 10, COX 17). Nuclear mutations may be general or specific and may be in protein required for assembly of holocomplex, mRNA binding protein (LRPPRC), SURF-1, SCO1, SCO2, and COX 10. Mitochondrial mutations may involve COX I, COX II, or COX III.

Complex V (Cx V)—ATP synthase (ATPase), according to Houstek et al. (2004) is composed of 14 nuclear DNA encoded subunits and two mtDNA encoded subunits. ATPase couples the proton gradient, generated by ETC (Cx I to Cx IV) to ATP synthesis. Mutations may be mitochondrial (ATPase 6) or nuclear DNA encoded (ATPase 12).

Respiratory Chain Activity

Literature presents a few studies in which respiratory chain activity has been correlated to FE. Even though FE was not the focus in a study by Briand et al. in 1981, the information

gained from that study may be applied to findings in new developments involving the study of the relationship between enzyme activity and FE in skeletal muscle. In that study with sheep, they observed that the slow twitch *spinatus* had low ATPase activity. Additionally, the fast twitch LM and semimembranosus had high ATPase, n-glycolytic and oxidative activity. The most glycolytic muscle of the study, *tensor fascia lata* and *semitendinosus* showed the highest ATPase activity. A direct study between respiratory chain activity and FE was completed by Bottje et al. (2002), who found respiratory chain activities in broiler breast and leg muscle to be higher in HFE compared to LFE birds. Igbal et al. (2004) extended findings by Bottje et al. (2002), reporting that LFE broiler breast samples were lower in mitochondrial complex activities than the HFE. More recently, Sandelin et al. (2005), in a study with samples from Angus cattle found that activities for all respiratory complexes (Cx I to Cx V) were higher in LFE compared to HFE steers. The differences between findings sparked the interest into species specific respiratory complex activities. Our study with gilts from the same genetic line produced different results from the above mentioned in that complex activities were found to be skeletal muscle dependent.

Protein expression

This section of our review was devoted to findings on protein expression in the skeletal muscle from various species to gain knowledge that may be useful in establishing a mechanism for the phenotypic expression of FE in swine from the same genetic line. Igbal et al. (2004) reported significantly higher protein expression, by Western analysis, in LFE poultry breast muscle for Cx III (cyt *b*, cyt *c1*, cor 1) and Cx IV (Cox II) compared to HFE birds. They concluded that the differential expression of the proteins could have been a compensatory

response to lower complex activities or increased protein oxidation in low FE. Earlier, Monemdjou et al. (2000), in a study with uncoupling protein-1 (UCP1) deficient mice, concluded that deviation in kinetics of mitochondrial proton leakage in skeletal muscle indicate a mechanism favoring the lean phenotype. Using laboratory rats, Obici et al. (2002) investigated a link between energy intake and energy expenditure. They showed that activation of the hexosamine biosynthesis pathway (HBP) rapidly decreased the expression of a cluster of nuclear-encoded mitochondrial genes involved in skeletal muscle phosphorylation. At the same time, the expression of UCP1 and the same mitochondrial genes was increased in brown adipose tissue. They reported that activation of HBP was replicated by overfeeding and concluded that a biochemical link to energy balance could be established. Leary (2003) confirmed that native holoproteins as well as cytochrome *c* content were associated with fiber type differences in red, white, and striated muscle from rainbow trout when parameters were expressed per mg mitochondrial protein. In a global gene expression study, Kong et al. (2011), concluded that HFE is associated with the upregulation of the anabolic process and the down regulation of fiber development, muscle function, cytoskeletal organization, and stress response. Polyana et al. (2017) reported findings of 73 differently expressed proteins in the mitochondria from Longissimus in Nelore steers. They found down regulation for xenobiotics and antioxidant metabolism with an upregulation of lipid oxidation and ketogenesis in HFE groups. Their study supported an earlier one (Kong et al., 2016) with liver tissue, revealing new genes and probable tissue specific regulators.

The cost of feed has dominated the topics for continued research in feed efficiency as presented in this review. However, Bottje and Carstens (2009), noted that not only cost of feed but also of fuel and fertilizer were of paramount interest. All three (feed, fuel, and fertilizers) as

they reiterated, along with increase in global demand for animal proteins, are products of biofuel policies.

Conclusion

As feed efficiency remains the most important factor in the production of commercial farm animals, the search for a mechanism or marker to elucidate the phenotypic expression of FE continues. To that end the focus of this review was on mitochondrial function in red (SpD) and white (LM) muscle types that differ in the abundance of mitochondria. The mitochondrion is responsible for energy production in cellular functions, including growth, which is the controlling factor in FE. The difference in abundance of mitochondria may be reflected in the respiratory chain activity and expression of certain mitochondrial proteins since the mitochondrion houses the ETC, site of oxidative phosphorylation. The ETC is responsible for ATP synthesis, with ROS production and mitochondrial apoptosis, to which inefficiencies might be attributed. Inefficiencies in the mitochondria may be assessed by measuring respiratory chain complex activities and protein expression. This literature review links FE to the mitochondrion as a function of muscle type, which may be reflected in respiratory chain activity and protein expression.

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Figure

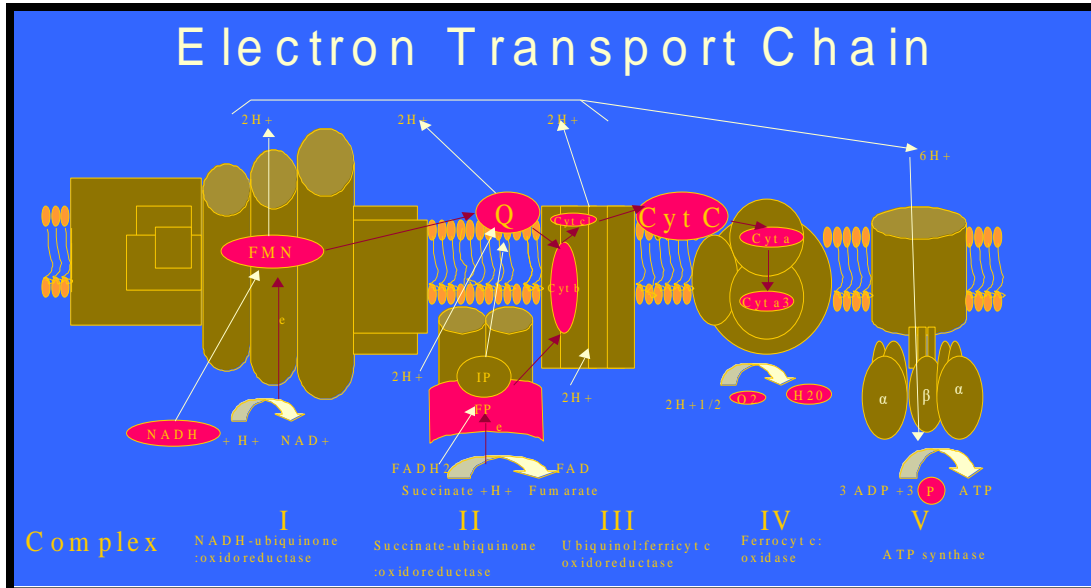


Figure 2-1. The Electron Transport Chain. This figure tracks the flow of protons and electrons involved in the production of ATP via the electron transport chain (ETC). The ETC is in the inner membrane of the mitochondrion. The electron transport system comprises four redox complexes (Cx I to Cx IV) and ATPase (Cx V) in addition to mobile electron carriers Q and Cyt c. The initial proton donor is NADH or FADH₂ and the final acceptor is oxygen to produce H₂O. The energy produced is used in the synthesis of ATP.

Moghadam et al., 2012

Chapter 3

RESPIRATORY CHAIN COMPLEX ACTIVITY IN *LONGISSIMUS* AND *SPINALIS DORSI* FROM HIGH AND LOW FEED EFFICIENCY SWINE WITHIN A SINGLE GENETIC LINE

Abstract

Phenotypic expression of feed efficiency (FE) has been linked to mitochondrial function, specifically respiratory chain complex activities in broilers fed the same diet and from the same genetic line. The objective of this study, therefore, was to determine if similar relationships between respiratory chain complex (Cx) activity and FE (gain:feed) are present in swine. Monsanto Choice Genetics furnished the pigs selected from 427 individuals within a single line using Feed Intake Recording Equipment. Seven of the pigs used in this study were from the top 1/6 of pigs in feed efficiency (HFE), and seven represented the bottom 1/6 in feed efficiency (LFE). A common commercial diet consisting of 15% protein, 4% fat and 6% fiber was fed prior to slaughter. Immediately after pigs were slaughtered, *Longissimus* (LM) and *Spinalis dorsi* (SpD) samples were collected from each of the 14 pigs. Mitochondria were isolated from LM and SpD by differential centrifugation. Mitochondrial respiratory chain complex activities were measured spectrophotometrically and reported in $\mu\text{M}/\text{mg P}$. Overall, the mean respiratory chain activities for high (H) and low (L) FE samples were higher in LM (19.11, H vs 17.32, L; SE = 1.46, 1.54, 6.76, 3.53 and 3.67 for Cx I – V, respectively) than in SpD (9.83, H vs 12.37, L; SE = 2.45, 2.78, 0.92, 0.94, and 0.48 for Cx I – V, respectively). Significant differences were observed between Cx I in LM (11.64, H) vs 4.55 (L), $P = 0.005$ and Cx II in SpD (17.96, H) vs 35.52 (L), $P = 0.0008$. Correlations between FE and enzyme activity were significant in LM and SPD for Cx I – II. The correlation between Cx I and FE was positive, whereas that with SpD

was negative, $C = 0.77$ and -0.75 , respectively, $P \leq 0.01$. The results of this study indicate a muscle type*dependent link between respiratory chain activity and phenotypic expression of feed efficiency in swine.

Introduction

Feed efficiency is one of the most important factors in the swine industry as feed cost represents 60-70% of the total expense for raising pigs to market weight. According to Ruusunen and Puolanne (2004), some fast-growing pigs reach market value up to 25 days sooner than slower growing pigs and size of skeletal muscles is an important indicator for growth. They reiterated that pigs generally are selected for their ability to grow rapidly, that intensive selection for fast growth in domestic pigs has precipitated a change in favor of light over dark muscle fibers and that the most economically important muscle is the LM. To our knowledge, the mechanism for such change has not been thoroughly investigated. Possible involvement of the mitochondria may be indicated since light muscle, abundant in glycolytic type fibers and low in mitochondrial number and capillary density, is dominant in fast growing pigs (Ruusunen and Puolanne, 2004). These findings suggest a decrease in oxidative type fibers, which are abundant in dark muscle, such as *spinalis dorsi* (SpD). Additionally, Bottje et al. (2002), in studies with a single genetic line of broilers, pioneered the research linking mitochondria function to the phenotypic expression of FE. They reported that mitochondria isolated from broiler breast meat of HFE Pedigree male broilers had higher complex (Cx) activity than mitochondria from LFE birds. In 2003, Lutz and Stahly presented similar findings in rats. Iqbal et al. validated these findings in 2004, suggesting that the differential expression of specific mitochondrial proteins genetically originating from mitochondria or nuclei (Table 3-1) may be a compensatory response to lower

complex activities of the electron transport chain. More recently, Grubbs et al. (2015) studied mitochondria from skeletal muscle and liver in pigs selected for residual feed intake (RFI). They found less reactive oxygen species (ROS) production in the white portion of the semitendinosus in the low compared with high RFI line using both NADH and FADH₂ as substrates. The mitochondria from the red portion had less ROS production when succinate was the energy substrate. Additionally, they reported a positive correlation between RFI and ROS in mitochondria from LM. These findings link phenotypic expression of FE not only to muscle type and mitochondrial function but also to the metabolic process by way of the feed intake (FI) consideration. These links may indicate the involvement of a process precipitated by reactive oxygen species (ROS) mediated defect in Cx I (Saada et al., 2004) or Cx II. (Senoo-Matsuda et al., 2003).

The objective of this study was to determine if a link could be established between the electron transport chain (ETC), muscle type and phenotypic expression of FE in swine, by measuring respiratory chain activities (Cx I to V) in skeletal muscle mitochondria from HFE and LFE animals within same genetic line and fed the same diet. Ultimately, the intent was to determine if the effect of FE on mitochondrial function as established in broilers, and other species was similar in porcine models.

Materials and Methods

Samples

The skeletal muscle samples were taken from 14 Monsanto Choice Genetics pigs selected from a group of 427 within the same genetic line and fed the same commercial diet consisting of 15% protein, 4% fat, and 6% fiber. Automatic Feed Intake Recording Equipment (FIRE) was

used to determine average daily feed intake (ADFI) in pen fed animals. Additional feed efficiency data included total feed intake (TFI), and total gain (TG). From these data, FE (gain:feed) and feed conversion ratio (FCR) were determined. Of the 14 pigs, 7 were in the top 1/6 in feed efficiency (HFE) and 7 from the bottom 1/6 (LFE). Immediately after the pigs were slaughtered, samples (~ 20 g each) were excised from *Longissimus* (LM) and *Spinalis dorsi* (SpD) muscles, frozen in liquid nitrogen, and stored at –80 °C until samples were analyzed.

Mitochondria Isolation

Mitochondria were isolated from the 14 LM and 14 SpD samples according to methods as described (Bhattacharya et al., 1991; Tang et al., 2002). Frozen LM or SpD muscle (~8 g) was thawed on ice, finely minced in isolation medium, incubated 5 min with 0.02% nagarase, and centrifuged at 1000 x g for 10 min. After discarding cellular debris, pellets were resuspended in 2 mL storage buffer, and frozen in liquid nitrogen for subsequent analyses.

To verify consistency of mitochondrial purity between feed efficiency groups (HFE and LFE), activity of citrate synthase as a mitochondrial enzyme marker was assessed by methods described by Srere (1969) and Zhang et al. (2001). The mitochondria samples were freeze-thawed twice -70 °C to disrupt mitochondrial membranes. The spectrophotometer was set at 412 nm, 30 °C, to mix 20 sec, and to read absorbance at 20 sec intervals. Absorbance of the reaction buffer (1M Tris-HCl: pH 8.0, (22 µL), 1 % Triton-X100 (22 µL), DTNB(44 µL) , d H₂O (117 µL), 9 mM AcCoA (µL) were combined with mitochondria (10-30 µg protein) for a total of 220 µL. Oxaloacetate (OAA, 5 µL) was added to initiate CS activity and followed for 3 min. A positive control was run simultaneously to determine the degree of non-enzymatic hydrolysis. Activity was calculated as a factor of change in absorbance after addition of OAA – change in absorbance before addition of OAA using an extinction coefficient of $\epsilon = 13.6 \text{ mM}^{-1}\text{cm}^{-1}$.

Mitochondrial protein was determined spectrophotometrically with a Bradford assay (Sigma Kit 610-A Sigma Chemical Co. St. Louis, MO). The protein assay solution (20 mL protein dye reagent + 80 mL d H₂O) was mixed. Then using 0.85% NaCl and the 300 mg/L protein standard included in the kit, 0.09, 0.15 and 0.30 mg/mL protein concentrations were prepared. Final volume for each sample and standard was 2.5 mL + 50 uL. Samples were incubated for ~ 2 min and protein concentration in mg/mL was read at 596 nm.

Respiratory Chain Complex Activities

Ultraviolet spectrophotometry was used to assess respiratory chain complex (Cx) activities. Activities are expressed in units of activity per min per mg of mitochondrial protein (U/mg P).

Complex I Activity. Complex I (NADH ubiquinone:oxidoreductase) activity was measured by methods of Galante and Hatefi (1978). Absorbance at 340 nm was monitored for 5 min with and without rotenone and rotenone-sensitive activity was determined by using 6.22 mM⁻¹ cm⁻¹ as extinction coefficient. Mitochondrial samples (25 to 40 µg protein) was incubated at 37 °C for 2 min in 176 µL of distilled H₂O. A reaction medium containing 50 mM Tris-HCl with 5 mg/mL BSA, pH = 8.0, 240 µM Potassium Cyanide (KCN), 4 µM antimycin A and 40 µM 2,6-dichlorophenolindophenol (DCPIP) was added and the reaction was initiated with the addition of 0.75 mM NADH. Absorbance was monitored for 3 min, then 4 µM rotenone was added to stop the reaction. Complex I activity was recorded for an additional 3 min and was quantified using the rotenone sensitive rate and an extinction coefficient of $\epsilon = 6.22 \text{ mM}^{-1} \text{ Cm}^{-1}$.

Complex II Activity. Complex II (succinate:ubiquinone oxidoreductase) activity was measured by following the secondary reduction of DCPIP by ubiquinone-2 (CoQ₂) at 600 nm for

5 min with and without TTFA as described by Barrientos (2002). An extinction coefficient of $21 \text{ mM}^{-1} \text{ cm}^{-1}$ was used. Mitochondria (25-40 μg) protein were added to a buffer containing 50 mM potassium phosphate (KH_2PO_4), 0.10 mM ethylene diamine tetra acetic acid (EDTA) and 18 mM succinate and incubated for 3 min. Rotenone (5 μM), 0.2 mM ATP, 100 μM DCPIP and 80 μM CoQ2 were added and the activity was recorded for 5 min and 1 mM thenoyltrifluoroacetone (TTFA) was added to inhibit Complex II activity. The specific activity of this complex was determined using the TTFA-sensitive rate and an extinction coefficient factor of $\epsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$.

Complex III Activity. Complex III (ubiquinol: ferricytochrome *c* oxidoreductase) activity was determined by measuring the rate of cytochrome *c* reduction by ubiquinol-2 as described by (Hatefi; 1978). The reduction of cytochrome *c* at 550 nm was recorded for 90 sec, and enzyme activity was calculated with an extinction coefficient factor ($\epsilon = 19.2 \text{ mM}^{-1/\text{cm}}$). Mitochondria (25-40 μg protein) was added to a medium containing 10 mM KH_2PO_4 , (pH 7.8), 2 mM EDTA, 5 mM MgCl_2 , 1 mg/mL BSA, 240 μM KCN, 4 μM rotenone, 200 μM ATP, 0.6 mM n-Dodecyl-B-D-malt side, and 40 μM oxidized cytochrome. Initiation of the reaction proceeded by adding 80 μM ubiquinol-2, as observed by a subsequent increase in absorbance followed for 90 sec. The reduction of cytochrome *c* with the addition of 4 μM antimycin A represented the non-enzymatic rate, which was subtracted for calculation of the specific activity of Complex III, using an extinction coefficient of $\epsilon = 19.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

Complex IV Activity. Complex IV (ferrocytochrome *c*: oxidoreductase) activity was determined by measuring the oxidation of reduced cytochrome *c* as a decrease in absorbance at 550 nm according to Galante and Hatefi (1978). The reduction of cytochrome *c* proceeded with the addition of a few crystals of sodium dithionite. The reaction medium contained 10 mM

KH₂PO₄, 250 mM Sucrose, 1 mg/mL BSA, 240 μ M KCN and mitochondria (25-40 μ g protein). The reaction was initiated by adding 10 μ M reduced cytochrome *c* to a final volume of 0.215 mL and the reaction was followed 60 sec. The activity was calculated using an extinction coefficient factor ($\epsilon = 19.2 \text{ mM}^{-1} \text{ cm}^{-1}$).

Complex V Activity. Complex V activity (ATP synthase) was measured according to methods of Barrientos (2002). Lactate dehydrogenase and pyruvate kinase were used as coupling enzymes. The assay was performed at 340 nm following the decrease in absorbance resulting from NADH reduction. The reaction medium (45 μ L: 50 mM Tris, pH = 8.0, 20 mM MgCl₂, 50 mM KCl, 15 μ M carbonyl m-chlorophenylhydrazide cyanide, 5 μ M antimycin A, 10 mM phosphoenolpyruvate, 2.5 mM ATP, 20 U lactate dehydrogenase, 20 U pyruvate kinase, 1 mM NADH and 5 mg/mL BSA) was incubated at 37 °C for 5 min. Mitochondria (25-40) μ g protein was added and incubated for 30 sec and the reaction was followed for 3 min. Oligomycin (3 μ M) was added and the reaction was followed for an additional 3 min. The oligomycin-sensitive ATPase activity was calculated with an extinction of $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

Statistical Analysis

The differences in respiratory chain activities between HFE and LFE mitochondria from LM and SpD were determined using ANOVA with a P value of ≤ 0.05 considered significant. The relationship between FE and respiratory chain activities was described as correlation coefficients generated by multivariate pair wise analysis. Interaction profiles were produced using response profiler to generate interaction plots. The SAS GLM procedures and standard least squares using full factorial model were used to generate means, SE, and significant differences (SAS Institute, Inc. Cary, NC).

Results

Animals Feed Efficiency Data

The animals from which samples were taken for this study were divided into two different ($P = 0.0001$) FE (gain:feed) groups as shown in Table 3-2 and Figure 3-1. Additionally, Table 3-2 shows significant differences ($P < 0.005$) in average daily (ADFI) and total (TFI) feed intake between the two FE groups (HFE and LFE). However, no differences ($P = 0.57$) in wt. gain were observed between FE groups. Feeding time in days was determined by the total feed (TFI) intake to average daily intake (ADFI) ratio to be 105 d. Subsequently average daily gain, as determined by total gain (TG) to 105 d, was similar ($P > 0.05$) in HFE and LFE pigs. Therefore, FI, with lean growth, could have been one of the controlling factors in this study.

Respiratory Chain Activities (Complexes I to V)

Means for respiratory chain complex (Cx) activities (I to V) in mitochondria from LM and SpD are recorded in Table 3-3 for HFE and LFE pigs. In the LM, Cx I activity was higher ($P = 0.005$) in mitochondria from HFE (11.64 ± 1.46) pigs than LFE (4.55 ± 1.46). Complex II activity, however, was higher ($P = 0.008$) in the LFE mitochondria than the HFE group in SpD but there was no difference in Cx II to Cx V activity in the LM between the two groups. Cx I, Cx III, Cx IV, and Cx V activity was similar between FE groups in the SpD mitochondria. Main effects mean for respiratory chain complex activities are also recorded in Table 3-3. Differences ($P < 0.01$) for all complexes between muscles are shown with higher Cx I activity in SpD compared to LM and higher Cx III to V in LM than SpD. In the FE groups, Cx I activity was higher ($P < 0.01$) in the HFE group compared to the LFE group (15.45 vs 9.47). An interaction

was observed for Cx II. The observed muscle by FE group interactions are depicted in Figure 3-2. This interaction for Cx II activity was due to a higher ($P < 0.01$) activity in the SpD muscle in the LFE group compared to the HFE group while the activity between the two feed efficiency groups was similar in the LM.

In Table 3-4, correlations between Cx activities and FE in porcine LM and SpD are shown. FE is positively correlated ($C = 0.41$; $P < 0.05$) to Cx I activity. Both Cx I and Cx II are negatively correlated to Cx III, ($P < 0.01$), and V ($P < 0.05$ and 0.01 , respectively). Complexes III and IV are positively correlated to Cx V ($P < 0.01$). Additionally, correlations between respiratory chain Cx activities and FE in the LM are recorded in Table 3-4. Here we observe a positive correlation ($C = 0.77$; $P < 0.01$) between Cx I and FE. Tendencies ($P < 0.20$) for positive correlations between complexes I and II with IV and V are also noted. Complex activities and FE correlations in the SpD are also shown in Table 3-4. A negative correlation ($C = -0.75$; $P < 0.01$) between Cx II and FE was noted. The table also depicts a positive correlation ($P < 0.01$) for Cx I and Cx V and also between Cx IV and Cx V ($P < 0.2$).

Discussion

Numerous studies in the 1970s and 1980s, according to Bottje et al. (2002) and Iqbal et al. (2004) provided evidence for a link between mitochondrial oxygen use and various dietary manipulations in different species. However, there were no reports regarding associations of mitochondrial function and feed efficiency within a single breed of animals fed the same diet. An additional effect of muscle type has been included since that time. Bottje et al. (2002) in a study with broiler breast and leg muscle found that electron leakage was greater in LFE than in HFE breast mitochondria. The leg mitochondria in that same study showed no differences in

basal electron leakage in leg mitochondria between FE groups. In their study, the activities of Cx I and Cx II were higher in HFE breast and leg muscle mitochondria compared to LFE mitochondria. They concluded that lower respiratory chain coupling in LFE muscle mitochondria may be precipitated by lower activities in Cx I and II and defects in electron leak, suggesting a cellular or molecular mechanism associated with phenotypic expression of FE. Complex I deficiency is the most commonly isolated defect of oxidative phosphorylation disorders, representing 1/3 of all cases respiratory chain deficiency (Saada et al., 2004). A defect in Cx I is additionally associated with decreased ATP production, increased oxidative stress, and accelerated apoptosis (Robinson, 1996). In 2004, Igbal et al. reported that complex I, II, III, and IV activities from broiler breast muscle mitochondria were higher in HFE compared with LFE birds. However, they found the expression of immunoreactive proteins to be higher in LFE samples for five proteins (core 1, *cyt c1*, *cyt b* (Cx III), Cox II (cytochrome *c* oxidase subunit II), and adenine nucleotide translocator (ANT1). In a study with Ghezel lambs, Sharifabadi, et al. (2011) studied low and high RFI lambs with no differences in ADG, but the low FCR sheep gained 70 g more per day compared to high FCR sheep. All five complex activities were higher in the low RFI compared to the high RFI LM samples. Complexes III, IV and V were lower in low FCR samples. In a study with broiler breast muscle, in 2009, Bottje et al. treated samples with guanosine or carboxyatractylate and observed that proton conductance was lower in HFE compared with LFE mitochondria. They thereby substantiated that membrane differences may also serve as a conduit to the phenotypic expression of feed efficiency. In comparing results to the information presented from studies with broilers and other species, we reported a link between FE and mitochondrial function in porcine samples from pigs within the same genetic line. Additionally, our findings indicated a muscle type link to the phenotypic expression of FE

that may be substantiated by comparing respiratory chain activities, and the connection to FI, and ROS production.

Samples and Feed efficiency data

Weight gain in HFE pigs may have been a combination of factors. Unlike the studies by Bottje et al. (2002) and Iqbal et al. (2004) total feed intake (TFI) and average daily feed intake (ADFI) differed significantly (Table 3-2) in the two FE groups, thereby adding an increase in calorie intake in LFE. The intake of more calories by LFE pigs may have been associated with enhanced production of reactive oxygen species (ROS) in the mitochondria possibly due to overeating and insulin resistance which could be normalized by calorie restriction (Civitarese et al., 2007). No difference (Table 3-2) in total gain (TG) was observed, but Whittemore (1993) stated that growth rate is the controlling factor in FE. Therefore, data revealing the time each pig reached market weight may have indicated that HFE and LFE were associated with fast growth and slow growth rates, respectively. However, a known in this study is that FI is a controlling factor. In Table 3-2, we noted also that total feed intake (TFI) was greater ($P = 0.0005$) in LFE (128.18 kg) compared with HFE (88.08 kg/I). At the same time, TG did not differ ($P = 0.57$) between HFE (44.88 kg/I) and LFE (43.21 kg/I) pigs. These findings are contrary to broiler studies, where feed intake (FI) did not differ between FE groups, but HFE birds gained more weight compared to LFE (Bottje et al., 2002). A newer approach to feed efficiency involving residual feed intake (RFI), evolved in swine studies, includes the backfat model as suggested by Patience et al. (2015). They reported low protein degradation, a decrease in oxidative stress as well as low electron leakage and reduction in ROS in low RFI muscle and liver mitochondria. In our study with porcine samples, no difference in ETC activity between HFE and LFE groups for

Cx III-V was observed. The only differences between groups were in Cx I (LM, $P = 0.005$) and II (SpD, $P = 0.0008$) activities and IP = 0.0001). Additionally, Cx activities between muscle groups were inversely proportional, moving from Cx I or II, then III to V, remarkably increasing in LM, and decreasing in SpD, respectively. These findings may suggest that low leakage may have occurred in LM, whereas high leakage occurred in SpD. In both broilers and gilts from the same genetic line and fed the same diet, a clear link to the phenotypic expression of feed efficiency as a function of mitochondrial oxidative phosphorylation has been established. However, a muscle type link to FE in swine offers new avenues to explore; the race is on and research is ongoing for that distinct pathway that is the dividing line between high and low FE.

Mitochondria Isolation and Protein Concentration

The quantity and relative purity of mitochondria isolated from swine LM and SpD as well as the HFE and LFE mitochondria verified that dark (i.e., SpD) muscle was highly concentrated in mitochondria compared to light muscle (i.e., LM). Mitochondria are responsible for aerobic respiration, crucial for energy production and play key roles in executing the apoptotic program (Kwong et al., 2007). Fast growing or high feed efficient pigs are at risk for increased ROS production (Kim and Kim, 2017) in mitochondria. As mitochondria are sites of ROS production and are at the same time vulnerable to damage as a result of ROS over production (Wei, 1998) and susceptibility of mitochondrial DNA (mtDNA) to deletions, mitochondrial inefficiencies may occur at the expense of electron leakage from ETC (Iqbal et al., 2004). This damage may be reflected in complex activity values.

Respiratory Chain Activities

Defects in either Cx I (Saada et al., 2004) or Cx II (Senoo-Matsuda et al., 2003) may lead to mitochondrial driven apoptosis. The mechanism of the process is conserved from nematodes to humans (Liu and Heingartner, 1999; Meier et al., 2000) to ameliorate oxidative damage (Senoo-Matsuda et al., 2003). They stated that apoptosis includes release of proapoptotic factors such as cytochrome *c* from the inner mitochondrial membrane to the cytosol, activation of caspases and ultimately disintegration of the defective cell and its preparation for phagocytosis (Figure 3-3). Anderson et al. (2006) in a study linking feed efficiency and apoptosis reported that H₂O₂ in type II fibers potentiate mitochondrial superoxide production and release and provide a potential mechanism for the development of mitochondrial dysfunction in skeletal muscle. Trade-offs to HFE may include an increased number of apoptotic events in selected oxidative muscle fibers with higher protein turnover in the rapidly growing skeletal muscles exposed to enhanced production of free radicals. With the eradication of these fibers or myonuclear sections in selected skeletal muscles, nutrients could be partitioned to the growth of light muscle fibers, which are prominent in HFE pigs (Ruusunen and Puolanne, 2004). Such occurrences would be expected if light muscle fibers (in LM, for example) were spared in the event dark muscle fibers such as SpD mitochondria were subjected to oxidative stress. This could lead to mitochondrial apoptosis, which negatively impacts cytochrome *c*, releasing it to the cytoplasm, thereby decreasing Cx activities for Cx III and IV in SpD. Ultimately, Cx V as well would be affected since the energy produced in electron transfer from Cx I or II to Cx III and then to IV determines ATP production at Cx V (ATPase) level.

Our study revealed a muscle group interaction for Cx II. However, both Cx I and II activities differed significantly between swine muscle type as well as FE group with Cx I activity

higher in HFE mitochondria compared with LFE in LM. Cx III to V activities were greater in LM compared to SpD. Complex II was higher in the LFE mitochondria than HFE in SpD. In the combined effects, Cx I and II activities were higher in SpD than LM, whereas activities for Cx III to V were higher in LM than SpD. In FE groups, Cx I activity was greater in HFE than LFE, whereas Cx II activity was higher in LFE, with most of that difference due to the higher activity in the SpD muscle.

Significant ($P = 0.01$) positive (0.77) and negative (-0.75) correlations in Cx I and Cx II, respectively between Cx activities and FE in LM and SpD are notable findings that may support Ruusunen and Puolanne (2004) suggestion of muscle type transformation in favor of light over dark muscle. In combined muscle groups, the significant positive correlations between Cx I activity and FE and the negative correlations between Cx I and Cx II, with Cx III and V indicates a defect involving Cx I and Cx II that may lead to release of cytochrome *c*, as suggested by Saada et al. (2004) and Senoo-Matsuda et al. (2003). They reported that destabilization of the mitochondria by the release of cytochrome *c* results in mitochondrial apoptosis. These findings indicate that in this Monsanto line of pigs the increase in LM may be at the expense the SpD. These results in conjunction with previous studies (Bottje et al., 2002; Iqbal et al., 2004; Ruusunen and Puolanne, 2004) intimately link FE to mitochondrial function via complex activities and suggest muscle type dependency for the phenotypic expression of feed efficiency.

In 2014, Bottje et al. in a follow up of Kong et al. (2011) findings, hypothesized the association of mTOR and protein degradation pathways for the phenotypic expression of feed efficiency. Kong et al. (2016) identified 228 mitochondrial proteins associated with HFE in Pedigree male broilers (PedM). Fernandez et al. (2019) investigated mitochondria in muscle from beef steers and noted complex II activity was higher in low RFI than in high RFI steers.

They reported that these findings suggest that skeletal muscle from low RFI steers have a greater number of mitochondria and more efficient coupling between respiration and phosphorylation compared with high RFI steers.

Summary

In summary, respiratory chain activities in our study were found to be muscle type dependent within swine FE groups. A visual depiction of the findings is presented in Figure 3-4, where interaction profiles are represented in the format of Cx activity (U/mg P) on the vertical axis by muscle group (left column), by FE group (center column) and by Cx I-V (right column). A higher activity was observed in the SpD muscle in the LFE group whereas activity was higher in the LM in the HFE group (Middle panel in the left column). Complex I and II activities appeared higher in SPD whereas complex III, IV, and V appeared higher in the LM (Lower panel in the left column). In the center column, a higher activity in LM was observed in the HFE group and the higher activity was observed in the SpD in the LFE group (Top panel in the center column). Cx I activity decreases from HFE to LFE; Cx II increases from HFE to LFE; no change in Cx III and Cx IV; and a decrease in Cx V activity from HFE to LFE (Lower panel in the center column). In the right column (Center panel), Cx II activity appears to be higher in LFE and lower in HFE and other complex activities appear similar. Complex I and II activity appear to be higher in the SpD muscle and Cx II, IV and V seem higher in the LM (Right column, top panel).

An interaction was established for Cx II, but not Cx I, and no significant differences between FE groups and Cx III, IV, or V activities were observed. Complex I activity was positively correlated with FE in LM, whereas Cx II activity was negatively correlated with FE in

SpD. These observations indicate a muscle type dependency between respiratory chain activity and phenotypic expression of FE in swine within a single genetic line.

Implications

As we observed in the summary and Table 3-3, significant effects ($P < 0.05$) included muscle type differences in respiratory chain activity for Cx I-V; differences between FE groups for Cx I and II; and an interaction due to the higher activity in LFE SpD compared with LM. Interestingly, activities in Cx III, IV and V were higher in LM compared with SpD. As a defect in Cx I or Cx II, in this case, in the SpD may lead to destabilization of mitochondria and consequently a decrease in Cx activities III-V due to cytochrome *c* leakage from the ETC. The higher LM activities suggest stability in the LM at the expense of the SPD. We conclude that the observations herein may be indicative of mitochondrial apoptosis involvement in the phenotypic expression of FE in swine within a single genetic line. According to Kwong et al. (2007), the respiratory chain is a modulator of apoptosis. Since mitochondrial apoptosis is intimately associated with the cytochrome *c* of the ETC, it appears that measurement of the respiratory chain activities and protein expression of the subunits would provide information that may be useful in determining if there is a connection between the ETC and the phenotypic expression of FE. Mitochondrial apoptosis may occur because of extensive damage by oxidants in the form of ROS or reactive nitrogen species (RNS). In this case, many apoptotic stimuli induce swelling, permeability and pore formation, dissipation of membrane potential. Cytochrome *c* is released from the mitochondria into the cytosol, forming an apoptosis initiating complex, the apoptosome. The complex is a combination of apoptotic protease activating factor-1 (Apaf-1), dATP, and procaspase-9. Active caspase-9 cleaves and activates caspase-3, which in turn activates a

cascade of caspases, leading to reorganization of the cytoskeleton. DNA replication and repair are shut down, DNA is destroyed, the nuclear structure is disrupted, and the cell disintegrates into apoptotic bodies, destroying the cell (Green and Kroemer, 1996; Green and Reed, 1998). Other mechanisms of interest for the phenotypic expression are the mTOR (mammalian target of rapamycin) and protein degradation pathways, which include measurement of parameters to evaluate the contribution of specific respiratory chain subunits to a pathway for the phenotypic expression of FE.

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Tables and Figures

Table 3-1. Genetic origin of respiratory chain complex (I to V) subunits.

	No. of Subunits	No. of Identified Mitochondrial Genes	No. of Identified Nuclear Genes
Complex I	30	7	34
Complex II	4	0	4
Complex III	11	1	10
Complex IV	13	3	10
Complex V	14	2	12
Total		13	70

Rotig and Munnich, 2003

Complex I (NADH coenzyme Q reductase)

Complex II (succinate-coenzyme Q reductase)

Complex III (reduced coenzyme Q-cytochrome *c* reductase)

Complex IV (cytochrome *c* oxidase)

Complex V (ATP synthase)

Table 3-2. Growth performance data for gilts within the same genetic line.

	TFI	ADFI	TG	ADG	FCR	FE, G:F
HFE	88.08	0.84	44.88	0.43	1.96	0.51
LFE	128.18	1.22	43.21	0.41	2.96	0.34
SE	6.08	0.06	2.06	_____	0.05	0.01
P Value, n=14	0.0005	0.0005	0.57	_____	0.001	0.0001

Growth period =105 d

HFE = high feed efficient; LFE = low feed efficient; TFI = total feed intake (kg.); ADFI = average daily feed intake (kg); FCR = feed conversion ratio; TG = total gain (kg); ADG=average daily gain; G:F = kg gain:kg feed intake

Table 3-3. Means for respiratory chain activities in $\mu\text{M}/\text{mg P}$

RC	LM		SE	P	SpD		SE	P	Significant Effects			
	HFE	LFE			HFE	LFE			MP	GP	IP	
Cx I	11.64	4.55	1.46	0.005	19.27	14.39	2.45	0.19	2.01	0.0002	0.007	
Cx II	8.32	6.88	1.54	0.51	17.96	35.52	2.78	0.0008	2.24	<0.0001	0.001	0.0001
Cx III	34.30	40.55	6.76	0.52	5.71	5.11	0.92	0.65	4.83	<0.0001		
Cx IV	14.54	12.29	3.53	0.66	3.36	4.10	0.94	0.60	2.58	0.001		
Cx V	26.76	22.49	3.67	0.39	2.87	2.75	0.48	0.86	2.41	<0.0001		

Main Effects

RC	Muscle		SE	P	Group		SE	P
	LM	SpD			HFE	LFE		
Cx I	8.10	16.83	1.42	0.01	15.45	9.47	1.42	0.01
Cx III	37.43	5.41	3.41	0.01	20.00	22.83	3.41	0.60
Cx IV	13.41	3.71	1.83	0.01	8.95	8.18	1.83	0.80
Cx V	24.63	2.81	1.70	0.01	14.81	12.62	1.70	0.40

RC—respiratory chain complex; Complex I (NADH coenzyme Q reductase); Complex II (succinate-coenzyme Q reductase); Complex III (reduced coenzyme Q-cytochrome *c* reductase); Complex IV (cytochrome *c* oxidase); Complex V (ATP synthase); LM—*Longissimus* muscle; SpD—*Spinalis dorsi* muscle; HFE—high feed efficient—from top 1/6 in feed efficiency of 427 pigs; LFE—low feed efficient—from bottom 1/6 in feed efficiency of 427 pigs; MP—muscle P value; GP—Feed efficiency group (HFE; LFE); P—P value; IP—muscle*group interaction P value; Cx II is excluded from Main Effects section due its having been included in significant Effects with an IP = 0.0001: $\mu\text{M}/\text{mg P}$ — $\mu\text{moles /mg}$ mitochondrial protein concentration.

Table 3-4. Correlations among respiratory chain Cx activities ($\mu\text{M}/\text{mg P}$) and FE in swine LM and SpD muscles

Muscle Group Combined					
	Cx II	Cx III	Cx IV	Cx V	FE
Cx I	0.48**	-0.50**	-0.20	-0.40*	0.41*
Cx II		-0.58**	-0.35+	-0.60**	-0.30
Cx III			0.53	0.70**	-0.09
Cx IV				0.51**	0.05
Cx V					0.08

LM					
	Cx II	Cx III	Cx IV	Cx V	FE
Cx I	0.44+	-0.12	0.45+	0.40+	0.77**
Cx II		0.26	0.51++	0.47++	0.19
Cx III			0.10	-0.01	-0.23
Cx IV				-0.03	0.13
Cx V					0.23

SpD					
	Cx II	Cx III	Cx IV	Cx V	FE
Cx I	-0.05	0.38	-0.07	0.70**	0.32
Cx II		-0.15	0.20	-0.25	-0.75**
Cx III			0.32	0.44+	0.19
Cx IV				0.06	-0.13
Cx V					-0.0002

**** P < 0.01; *P<0.05; ++P<0.10; +P<0.20; n=14.**

LM--*Longissimus* muscle; SpD—*Spinalis dorsi*; CMG—Combined Muscle Groups—LM and SPD

Complex I (NADH coenzyme Q reductase)

Complex II (succinate-coenzyme Q reductase)

Complex III (reduced coenzyme Q-cytochrome *c* reductase)

Complex IV (cytochrome *c* oxidase)

Complex V (ATP synthetase)

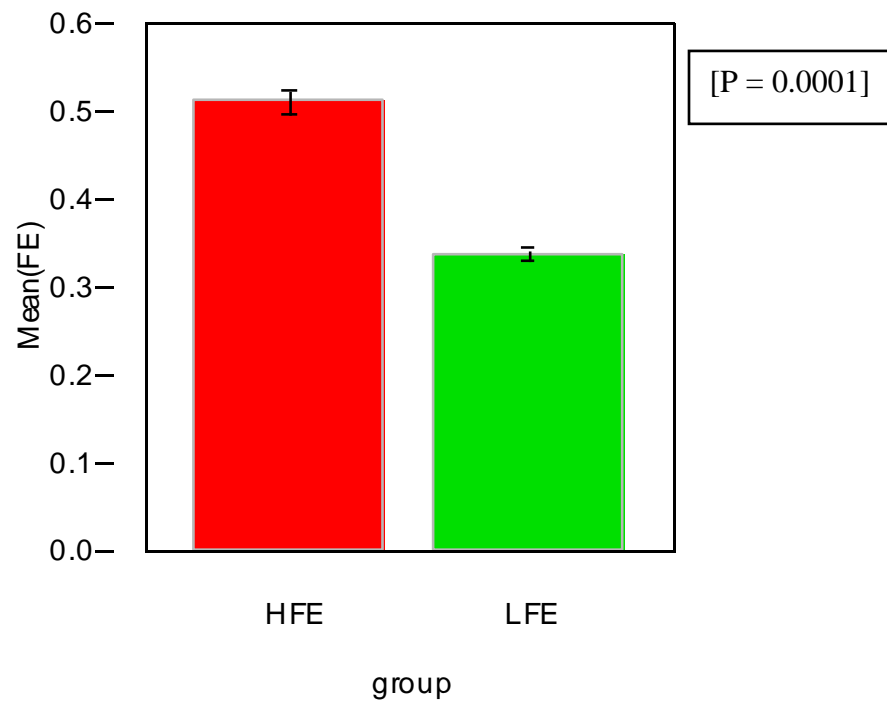


Figure 3-1. Feed efficiency data for HFE and LFE swine within the same genetic line. HFE, P= 0.0001; n =7; LFE, P =0.0001, n = 7; SE = 0.01

HFE=high feed efficiency; LFE=low feed efficiency

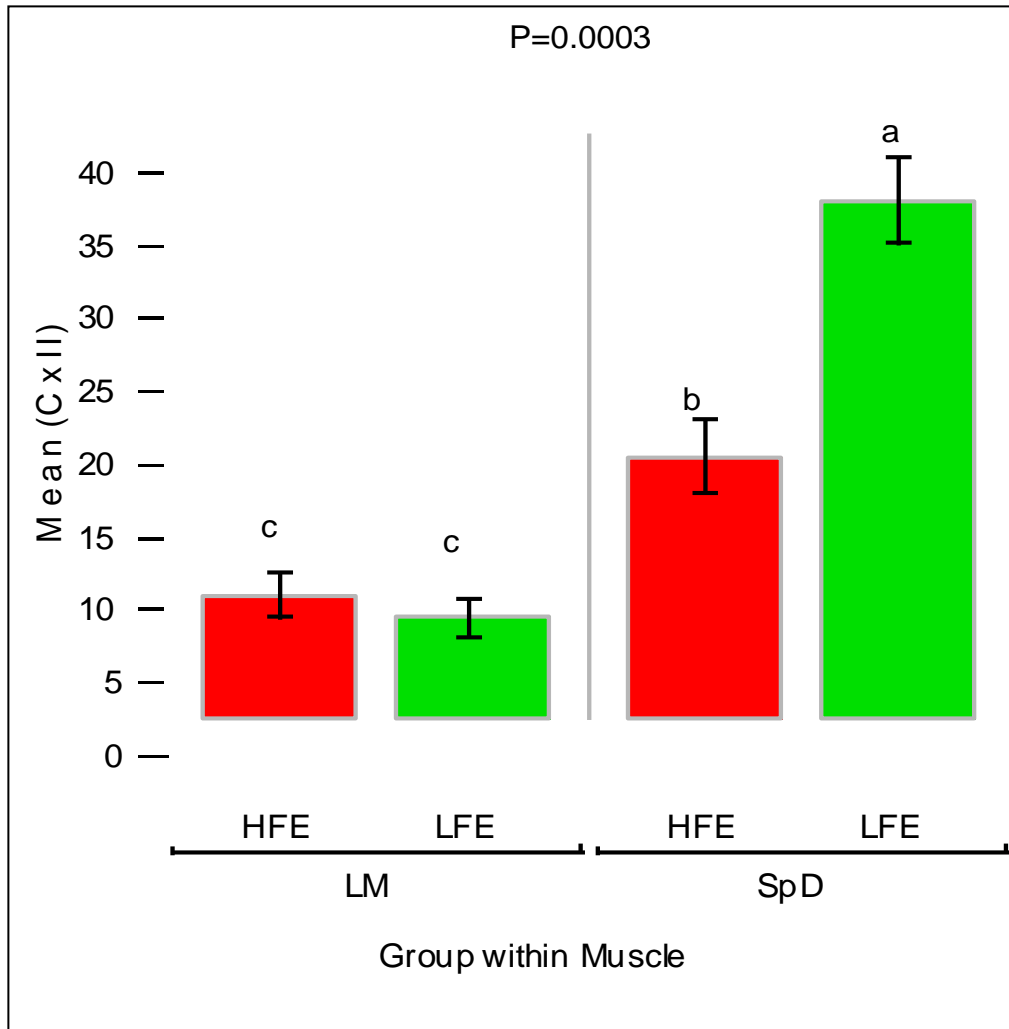


Figure 3-2. Muscle by group interaction ($P < 0.01$) for Cx II (respiratory chain activity means in units/mg of mitochondrial protein ($\mu\text{M}/\text{mg P}$)).
LM HFE mean = 8.32 ± 1.46 , $n=7$; LM LFE mean = 6.88 ± 1.46 , $n=7$;
SpD HFE mean = 17.96 ± 2.78 , $n = 7$; SPD LFE mean = 35.52 ± 2.78 , $n = 7$.

LM—*longissimus* muscle; SpD—*spinalis dorsi* muscle

HFE—high feed efficient = 7 samples from the top 1/6 in feed efficiency of 427 pigs
Monsanto Choice Genetics

LFE—low feed efficient = 7 samples from the bottom 1/6 in feed efficiency of 427 pigs
from Monsanto Choice Genetics.

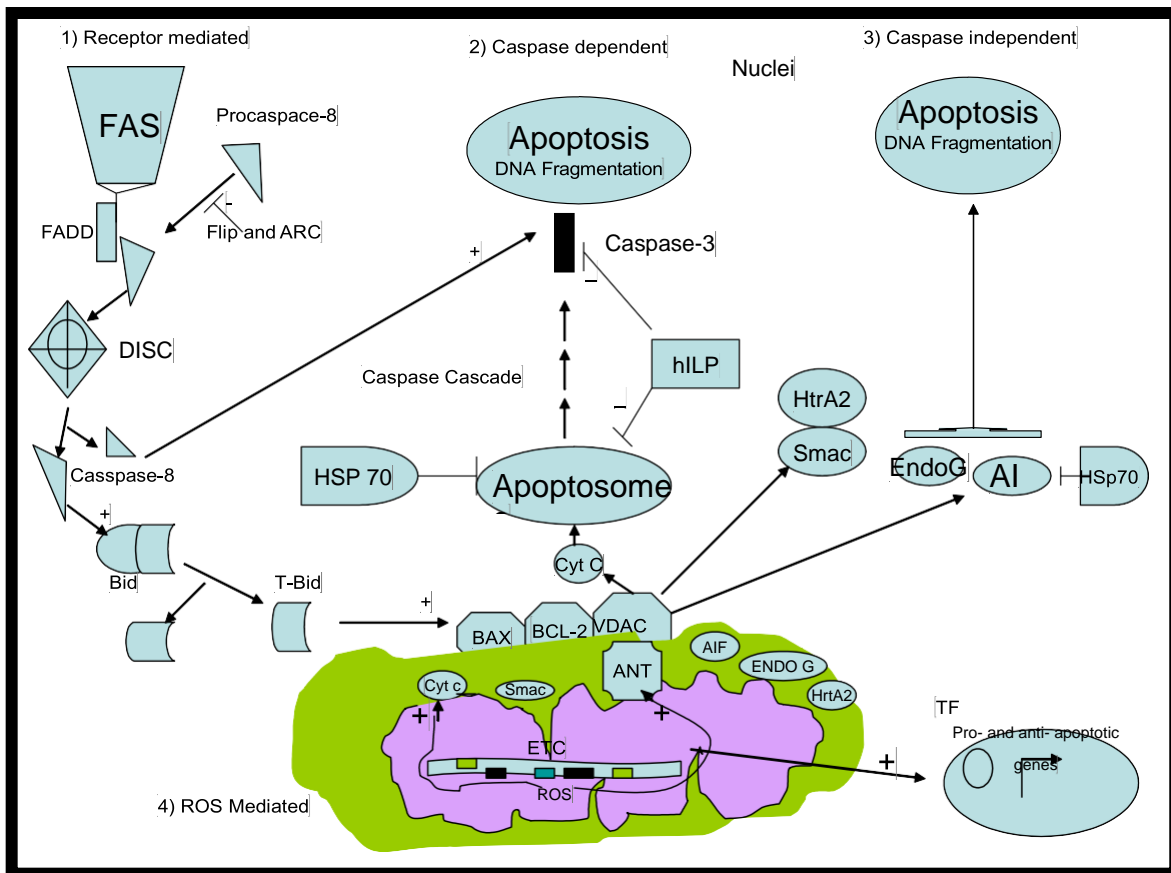


Figure 3-3. Mechanisms of apoptosis in skeletal muscle. This figure shows that apoptosis, which occurs in normal development and is conserved from nematodes to humans, may be caspase dependent or caspase independent. Caspase dependent apoptosis is either receptor (caspase-8) or ROS (caspase-9) mediated, involving the release of cytochrome *c* to the cytoplasm for the formation of an apoptosome and caspases in the production of DNA fragmentations.

AIF—Apoptosis initiating factor; ANT—adenine nucleotide translocator; ARC—Apoptosis repressor with CARD domain; BAX—bcl-2 associated protein X; BCL-2—antiapoptotic family of proteins (first found in B cell leukemias). Bid and t-Bid (BH3 only proteins interacting domain death agonist); Caspase—intracellular protease; DISC—death inducing signaling complex; Endo G—Endonuclease G; FADD—Fas associated death domain; Fas—expressed on the surface of cells and indicate death signal; Flip—FLICE inhibiting protein; HLP—hepatocyte growth factor-like protein; HSP—heat shock protein; HtrA2—high temperature requirement protein A2; ROS—reactive oxygen species; Smac; VDAC—voltage dependent anion channel.

(Adhihetty and Hood, 2003)

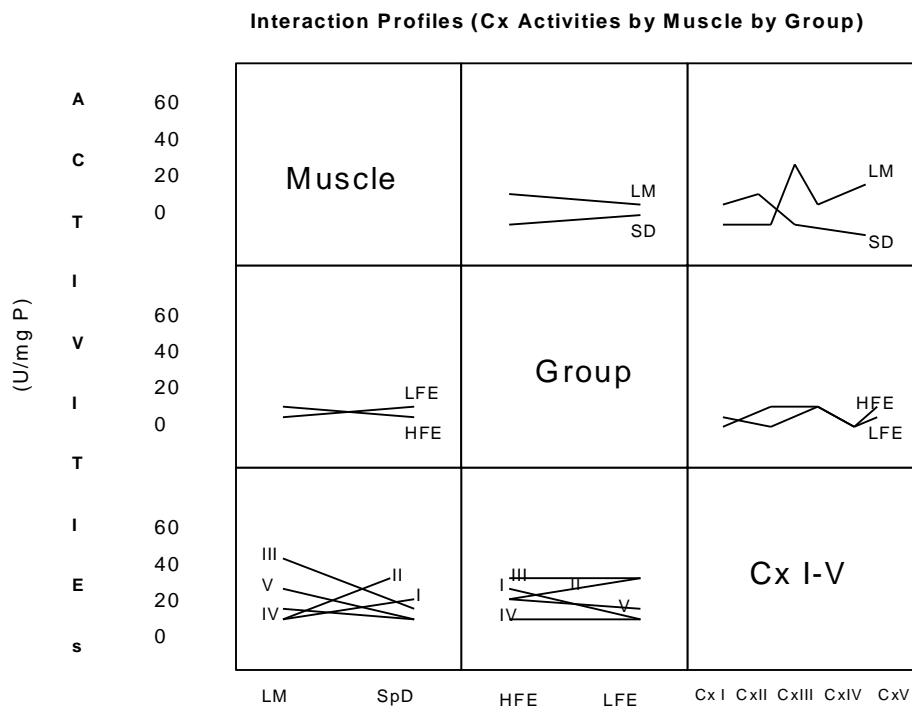


Figure 3-4. Summary chart for respiratory chain activities muscle*type FE group interactions. The left column depicts overall feed efficiency group activities within muscle interactions; the center shows overall muscle group activities within feed efficiency group: the right column presents individual points representing activities (U/mg P) for each complex (I to V) for each muscle and each feed efficiency group. Lines that cross and are parallel represent interactions and non-interaction, respectively.

LM—*Longissimus*; SD = SpD—*Spinalis dorsi*; HFE—high feed efficient; LFE—low feed efficient; I, II, III, IV, and V—complexes I to V, respectively; U/mg P—units/mg mitochondrial protein.

Chapter 4

PROTEIN EXPRESSION AND OXIDATION IN MITOCHONDRIA ISOLATED FROM HIGH AND LOW FEED EFFICIENT *LONGISSIMUS* AND *SPINALIS DORSI* MUSCLES FROM SWINE WITHIN THE SAME GENETIC LINE

Abstract

Feed efficiency in swine remains the most important consideration in raising pigs to market value due to the 50-70% cost of feed for the process. Mitochondrial function via respiratory chain complex activity has been both negatively and positively correlated to FE and linked to protein expression in broilers and growth of skeletal muscle in pigs. The objective of this study was to determine if protein banding patterns, protein carbonyls, and differential expression of selected mitochondrial proteins show similar relationships in skeletal muscle mitochondria between high and low feed efficiency animals within the same genetic line as previously observed in poultry and other species. Animals were selected from the same genetic line and included seven pigs from the top 1/6 as high feed efficient (HFE) and seven from the bottom as low feed efficient (LFE) from 427 Monsanto Choice Genetics. Immediately after slaughter, samples were excised, frozen in liquid nitrogen and stored at -80 °C until analyses were completed. Methods included centrifugal isolation of mitochondria; UV spectrophotometric determination of protein concentration (U/mg P); 1D and 2D gel electrophoresis for banding patterns and pixel intensities; and western blots for determination of carbonyls and selected immunoreactive proteins from subunits of the respiratory chain. The 1-D gel electrophoresis revealed 18 protein bands in both LM and SpD. Significant ($P \leq 0.05$) differences in pixel intensities were observed at bands 1-15 between LM and SpD, whereas no

significant ($P \geq 0.05$) differences were detected between HFE and LFE samples. However, muscle by treatment interaction approached significance ($P = 0.07$). Pi differed significantly ($P < 0.01$ and $= 0.02$ for Cox IV and ATPase α , respectively) between FE groups in LM. Ten protein subunits were significantly ($P \leq 0.05$) higher in pixel intensities in LM compared with SpD. Significant ($P \leq 0.02$) differences were observed between HFE and LFE in Cox I (130.18 vs 76.07 and ATPase (104.42 vs 80.17), respectively. Interaction profiles were noted (IP = 0.18, 0.08, 0.18 for CxI 30, Cox II and Cox IV, respectively). Means for protein carbonyls (Car I and II, respectively) in LM (116.70 and 125.93) mitochondria were lower ($P < 0.01$) than in SpD (121.16 and 139.01). Interactions ($P = 0.04$ and 0.02) for protein oxygenation were observed for Car I and II, respectively. Positive correlations to FE were observed in Cox II (in combined muscle groups), Cox IV and ATP α (in LM); Cox II and ATP α (in SpD). Additionally, CX II 30 was negatively (-0.66,) correlated to FE. Car I and II were negatively correlated to FE in SpD. The 2D gel with the least square mean revealed significant ($P \leq 0.05$) differences in number of protein spots in LM between HFE (882, b) and LFE (1057, a) as well as between HFE (273, c) and LFE (144, d) in SpD. Pixel intensities as fold differences were observed for 21 protein spots separated by gel electrophoresis. Seventeen of 21 were LFE phenotype in LM whereas in SpD 18 of 21 were HFE. Ten and 2 proteins are muscle and FE linked, respectively. Four of the protein subunits are associated with muscle and FE interactions in addition to carbonyls link to muscle type interaction. Therefore, the mitochondrial link to FE and muscle type has been established in this group of pigs from the same genetic line.

Introduction

The controlling factor in feed efficiency (FE) according to Whittemore (1993) is growth

rate. However, our study with gilts from the same genetic line and fed the same diet for lean muscle development did not produce pigs with divergent weight gain. Feed intake (FI) differed significantly and low FI could have stimulated compensatory growth (Heyer et al., 2007) of skeletal muscles. Therefore, our study assumes that high and low feed efficiencies are associated with fast and slow growth rates of lean muscle, respectively. Ruusunen and Puolanne (2004) stated that pigs are selected for their ability to grow rapidly and fast growth is associated with increased production of reactive oxygen species (ROS) in skeletal muscles. Increased ROS may induce oxidation and mitochondrial defects in proteins and other biological molecules (Wlodek et al., 2001). A combination of these events may precipitate differential banding patterns and protein expressions (Pumford et al., 2004) in skeletal muscle mitochondria and suggest pathways for the elucidation of a mechanism for the phenotypic expression of FE. In 2005, Pumford et al. presented results showing that HFE and LFE broilers have differences in banding pattern intensities in mitochondria from HFE and LFE broiler breast meat. Iqbal et al. (2004) reported that protein carbonyls, an indicator of oxidative stress, were 81% higher in mitochondria isolated from broiler breast muscle of LFE birds than HFE broilers.

According to Stadtman and Berlett (1998), oxidatively modified proteins are produced by ROS and may involve propagating radicals and a variety of ROS and stable products. In 1999, McCall and Frei revealed that protein oxidation markers include protein carbonyl derivatives, oxidized amino acid side chains, protein fragments, and formation of advanced glycation end products. Dean et al. (1997) and Hazen et al. (1997) reported that protein carbonyl derivatives are generated by the oxidative cleavage of the peptide main chain or by oxidation of arginine, lysine, proline, and threonine. Wlodek and Zeisel (2001) added that during lipid peroxidation, unsaturated aldehydes may form Michael addition adducts with cysteine, histidine, and lysine of

proteins producing carbonyl derivatives. As fiber type and respiratory chain activity from swine skeletal muscle mitochondria have been inextricably linked to the phenotypic expression of FE, the objective of this study was to examine the molecular basis of mitochondria isolated from *Longissimus* (LM) and *Spinalis dorsi* (SpD) muscle of high (HFE) and low (LFE) feed efficient swine.

Specific objectives

- 1) To determine if protein banding patterns differed in mitochondria from swine LM and SpD muscles and FE group within the same genetic line
- 2) To evaluate swine LM and SpD mitochondria for degree of protein oxidation (i.e., carbonyls) and the association with the phenotypic expression of FE
- 3) To assess the difference in protein expression of selected subunits in respiratory chain complexes from mitochondria in LM and SpD of HFE and LFE swine within the same genetic line

Materials and Methods

Samples

Monsanto Choice Genetics supplied the samples for this study. From 427 pigs within the same genetic line, seven were selected from the top (HFE) 1/6 in FE and seven from the bottom (LFE) 1/6 in FE. Feed intake was determined by Feed Intake Recording Equipment (FIRE). Additional information recorded included total weight gain, feed intake and feed efficiency. Immediately after slaughtering, 14 LM and 14 SpD samples (~50 g each) were excised, frozen in liquid nitrogen and stored at -80 °C until analyses were completed.

Isolation of mitochondria

Approximately 8 g of frozen samples was allowed to thaw on ice for about 45 min and then finely minced in isolation medium A (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl, and 46 mM KCl, pH 7.4) and incubated at room temperature in 50 mL of isolation medium containing nagarase (Sigma chemicals Co., St Louis, MO). Mitochondria were isolated as described by Bhattacharya et al. (1991) with modifications by Tang et al. (2002) from swine LM and SpD. Approximately 8 g of LM or SpD was finely minced and homogenized in a Potter-Elvehjem vessel with a Teflon pestle of 0.16 mm clearance (Thomas Scientific, Swedsboro, NJ.), centrifuged at 1000 x g for 10 min, and strained through a double layer of cheese cloth. Mitochondria were isolated from homogenate following an additional 5 min incubation on ice while stirring. After cellular debris was discarded, the supernatant was centrifuged (10,000 x g 15 min) to obtain to secure the mitochondrial pellet. The pellet was rinsed and washed in 10 mL of isolation medium A plus 0.5% BSA (without nagarase). Mitochondria were pelleted by centrifugation (8000 g x 15 min) in incubation medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, and 5 mM KH_2PO_4 , pH 7.4). The final pellets were resuspended in 1-2 mL of incubation medium and frozen in liquid nitrogen for subsequent biochemical analyses. Protein concentration was determined with a Bradford assay (Sigma Kit 610-A, Sigma Chemical Co. St. Louis, MO.)

SDS-PAGE

Proteins were separated with SDS-PAGE on 10% and 12% gels for banding patterns and western blotting in 1D and 2D analysis, respectively. The 10% gels were prepared by methods as described by Laemmli (1970) and proteins were transferred onto polyvinylidene difluoride

(PVDF; VWR Scientific Products Co., West Chester, PA.) in a submerged system using Hoefer transfer units (Hoefer, San Francisco, CA) (Pumford et al., 1990).

Protein Banding Patterns

One dimensional gel electrophoresis. Using SDS-PAGE 10% polyacrylamide mini gels submerged in Tris-HCl buffer (0.025 M Tris, 0.20 M glycine, 3.5 mM SDS, pH ~ 8.3 with HCl) proteins were separated using a Hoefer electrophoresis mini gel system at 200 V for approximately 2 hr. The gels were stained with Coomassie blue (R-250, Molecular Probes Inc., Eugene, OR.) for 1 hr. The gels were then destained with methanol-acetic acid to obtain appropriate contrast and scanned for pixel intensities using Agfa (Arcus II) scanner (Agfa-Gevaert, NV).

Two-Dimensional Gel Electrophoresis. The first dimension consisted of isoelectric focusing using Amersham Pharmacia Immobiline by Amersham Pharmacia. The immobiline Drystrip gels are comprised of preformed pH gradient immobilized in a homogenous polyacrylamide gel and are purchased dried cast on a plastic backing in packs of 12 strips. The strips were rehydrated in the Ettan IPGphor strip holder in rehydration buffer (8 M urea, 2% CHAPS, a few grains bromophenol blue, 25 mL, double distilled H₂O) stored in 3 mL aliquots at -20 °C. IPG Buffer (8 mg of DTT per 3 mL of rehydration buffer) were prepared just prior to use.

Stock SDS equilibration buffer (50 mM Tris-HCl, pH 8.8; 6 M urea, 30% glycerol, 2% SDS, 200 mL bromophenol blue, Millipore H₂O) was prepared and stored in 30 mL aliquots at -20 °C. Prior to use, DTT was added to the equilibration buffer. Agarose sealing solution (SDS running buffer, 0.5% agarose, 0.002% bromophenol blue) was prepared in a 500 mL beaker,

heated while stirring, until agarose completely dissolved, and was then dispensed in 2 mL aliquots into screw-cap tubes and stored at room temperature.

Samples were prepared for 50 ug final concentration by the following equation:

Sample concentration * Sample volume = final concentration * final volume using 125 uL rehydration solution. Sample (125 uL) in rehydration solution was pipetted into an IPGphor Strip Holder. The plastic back was removed by peeling the immobilized dry strip (Appendix XI, p. 111) starting at the blunted end while holding with flat tweezers. The blunt end was numbered with sample number using a very- fine- tip marker. The immobilized dry strip (Appendix XI, p. 111) was placed in tray channel with gel side down and the anode (+) at pointed end of the Strip Holder. Air bubbles were avoided, and all tips of gels were lined up to same corresponding spots in the Strip holder. Each Strip was overlaid with PlusONE DryStrip Cover Fluid starting at the anode end and working toward cathode. Gels were rehydrated for 10 hrs.

Equilibration buffer was prepared by splitting 30 mL of stock equilibration buffer and adding 150 mg DTT per 15 mL SDS equilibration buffer. Each IPG Strip was gently removed from holder, rinsed with Millipore water, and placed in a 10 mL polypropylene tube with support film toward wall. DTT-containing solution (2 mL) was added, and the tube was capped and placed on a rocker for a 15 min calibration.

The mini-gel Hoefer SE260 electrophoresis unit was set up and ready for placement of the IPG Strip and sealing solution was warmed to 100 °C, while molecular weight markers were heated for 3 min at 100 °C. Using SDS-PAGE 12% polyacrylamide mini gels, proteins were separated by 2D electrophoresis. The Hoefer SE 260 ran at 100 V for ~ 15 min for the stacking period then, 200 V for the remainder until the dye front was approximately 1 mm from the bottom of the gel. After electrophoresis, the gels were removed and stained with a Silver Quest

Staining Kit by Amersham Biosciences (Berkelman and Stenstedt, 1998).

Protein Carbonyls

The expression of protein carbonyls was measured in pixel intensities using chemiluminescence spectrophotometry and The Scion Image Software (Scion Corporation, Chicago, IL). Fessard and Livingstone (1998) described a method for tagging potential oxidatively damaged proteins by derivatization of carbonyls using 2,4-dinitrophenylhydrazine. Western blots were used for the detection and proteins were separated on SDS-PAGE onto 10% mini gels, transferred onto PVDF membranes, and incubated in 1 volume of 20 mM 2,4-dinitrophenyl hydrazine in 10% trifluoroacetic and 2 volumes of 12% SDS. After 15 min, 1.5 volumes of 2 M Tris-base was added and further incubated for 20 min. Blots were developed as described under **Immunoblots for Mitochondrial Protein** using anti-dinitrophenyl antiserum (1:1000 dilution) from Sigma Chemicals Co., St Louis, MO, and a peroxidase based chemiluminescence assay (Agilent Technologies, Inc. Santa Clara, CA).

Immunoblots for Mitochondrial Protein

Specific primary antibodies were used to develop western blots for respiratory chain protein subunits and protein using a peroxidase-based chemiluminescence detection system. Blots were blocked with a blocking agent and incubated overnight at 4 °C in 10mM Tris, and 0.02% sodium ethyl mercuri-thio-salicylate (thimerosal, pH 7.6). The primary antibodies were purchased from Molecular Probes. Dilutions for the primary antibodies are provided in Appendix I. After incubation with primary antibodies, blots were washed with detergent buffer (0.5% casein, 150 mM NaCl, 10 mM Tris 0.02% thimerosal, 0.1% SDS, 5% Triton X-100) and

2x with washing buffer (0.5% casein, 150 mM NaCl, 10mM Tris for 5 min each, and rinsed with distilled deionized H₂O 3X before and after incubation with detergent or washing buffers. Blots were incubated for 90 min with appropriate peroxidase-labeled secondary antibodies (goat anti-mouse Gig (Sigma Chemicals Co., St. Louis, MO) and then washed with detergent washing buffers, and Tris-saline. Blots were treated with substrate (Super Signal West Durra Extended Duration; Pierce, Rockford, IL) for 5 min, and chemiluminescence bands were detected using a charge-coupled device (CCD) camera (Fuji LAS 1000plus; Fuji Photo Co., Ltd., Tokyo, Japan). The molecular weights of separated proteins were estimated by comparison with ProSieve color molecular weight standards (Bio-Whittaker Molecular Applications (BMA), Rockland, ME).

Statistical Analysis

The differences in FE group between HFE and LFE were analyzed using ANOVA for total wt gain:feed intake. The relationship between FE and underlying components of FE were studied with multivariate pairwise correlations. Results for protein expressions in LM and SpD muscles from HFE and LFE swine were analyzed using a full factorial standard least squares model with Student's t-Test to determine significance with $P \leq 0.05$, unless otherwise stated, used for differences (SAS Statistical Institute, Cary, NC, 2003).

Results

Feed efficiency (FE) data for gilts in this study are provided in Table 4-1. The FE ratios (Gain:feed) in kg/I, $P = 0.0001$) were 0.51 and 0.34 for HFE and LFE, respectively. Total feed intake (TFI in kg) and average daily intake (ADFI in kg) for HFE (88.08 and 0.84) vs LFE (128.21 and 1.22) pigs differed significantly ($P = 0.005$) as well. Feed conversion ratios (FI/G in kg) were also different ($P = 0.0005$) with 1.96 and 2.96 for HFE and LFE, respectively. Total

gain was similar ($P = 0.57$) between the two FE groups, HFE = 44.88 and LFE = 43.21 kg/I.

We began our study with banding patterns (Table 4-2) where the 1-D gel electrophoresis revealed 18 protein bands in both LM and SpD. Significant ($P \leq 0.05$) differences in pixel intensities (P_i) were observed at bands 1-15 between LM and SpD whereas no significant ($P \geq 0.05$) differences were detected between HFE and LFE samples. However, muscle by treatment interaction approached significance ($P = 0.07$). Continuing to a more specific protein search, we compiled the list in Table 4-3 of immunoreactive proteins selected as an investigative tool for measuring protein intensity (P_i). Table 4-4 lists the results of immunoblots measuring P_i . Starting on the left, we observe that both Cox IV and ATPase α differed significantly ($P < 0.01$ and $= 0.02$ for Cox IV and ATPase α , respectively) between FE groups. Main effects means for protein subunits revealed significant ($P \leq 0.05$) differences between P_i in immunoreactive proteins Cx I 15 (58.92 vs 4.28); Cx I 17 (87.58 vs 28.73); Cx I 30 (91.96 vs 16.01); Cx II 30 (137.22 vs 24.76); Cor I (91.93 vs 15.77); FeS (71.68 vs 20.97); Cox II (118.41 vs 60.59); Cox IV (140.34 vs 65.92); ATPase α (109.93 vs 74.65) and ATPase β (115.32 vs 58.15) in LM and SpD, respectively). Means for protein carbonyls (measure of protein oxygenation) are listed at the bottom of the table as Car I and II. In LM, P_i was lower ($P < 0.01$; 116.70 and 125.93) than in SpD (125.93 and 139.01) for Car I and II, respectively. Muscle by treatment interactions ($P = 0.04$ and 0.02) for protein oxygenation were observed for Car I and II, respectively. Next, Table 4-5 (P_i) shows significant ($P \leq 0.05$) correlations between selected immunoreactive proteins (P_i) and FE. In the combined muscle group, $C = 0.68$ for Cox II. In LM the correlations were significant ($C = 0.50$ and 0.41) for Cox IV and ATP α , respectively. In SpD, correlations in Cox II ($C = 0.58$) and ATP α ($C = 0.78$) were positive, whereas P_i in Cox II 30 negatively ($C = -0.66$) correlated with FE. Additionally, in SpD, Car I and II, P_i and FE were negatively correlated ($P \leq$

0.05, $C = -0.56$ and -0.53 , respectively).

The overall results rendered ten differently expressed immunoreactive proteins with higher Pi in LM compared to SpD. Two (Cox IV and ATPase α) of the proteins were significantly ($P = < 0.01$) different between FE groups. Three (Cx I 30, Cox II and Cox IV) caused approaches to significant interactions. Carbonyls (Car I and II) were higher in SpD compared to LM. Muscle by treatment interactions ($P = 0.04$ and 0.02) were observed for Car I and Car II, respectively.

In order to visualize our findings, we proceeded from the general to the specific in search of protein indicators in one- and two- dimensional-gel electrophoresis for visible patterns to track as we continue our investigation of biomarkers for the phenotypic expression of FE. Figure 4-1 is a depiction of banding patterns in mitochondrial proteins. We observed the pattern of separation in each muscle type and FE group and were able to gain an understanding of and to visualize (Figure 4-1) the pattern of separation (Figure 4-2) of mitochondrial proteins via gel electrophoresis. Two muscle groups presented revealed 18 distinct bands and approached muscle type interaction for significance ($P = 0.07$) at band 7. Bands 1-15 differed significantly ($P \leq 0.05$) different in pixel intensities between LM and SpD. In the HFE and LFE groups, however, no significant ($P > 0.05$) differences were observed. Figure 4-3 is a two- dimensional gel electrophoresis representation of mitochondrial protein banding patterns in HFE and LFE from swine LM and SpD. The greatest number of protein spots (counted manually in three replications) appeared in the LM LFE (1057 spots), followed by LM HFE (882), then SpD HFE (273) and the least in the SpD LFE (144). The total number of spots differed ($P < 0.05$) among both FE and muscle groups (Figure 4-5). In Figure 4-3, mitochondrial protein from HFE and LFE LM and SpD is expressed by spots in gel associated with pixel intensities for specific

molecular weight (MW), and isoelectric point (pH). Twenty-one spots were randomly selected for a range of isoelectric points from pH 6.0 to 9.2 and MW from 35 to 130. In the LM 17 of the 21 spots were LFE proteins with only 4 HFE spots. In SpD, 18 were HFE spots whereas 3 were LFE spots. The 3 figures presented herein serve as visuals in support of the results which have established a muscle type link to FE via protein expression data. As with respiratory chain activities, these results support the concept of a muscle dependent link to mitochondrial function and FE.

Discussion

Various studies from the 1970s and 1980s according to Iqbal et al. (2004) have substantiated the relationship between FE and mitochondrial function. More recent findings by Bottje et al. (2002); Iqbal et al.(2003); Ojano-Dirain et al. (2003) in broilers, Lutz and Stahly (2003) in rats and others in rainbow trout (Silverstein, 2006) were some of the species in phenotypic expression of FE in relation to mitochondrial function had been investigated. In 2004, Bottje et al. clearly established the FE-mitochondrial link in a study on mitochondria from broilers within the same genetic line and fed the same diet. They found lower ROS and electron leakage in the HFE mitochondria compared to the LFE with increased protein oxidation in LFE muscle mitochondria is due to site-specific leaks. They concluded that lower ROS, and tighter coupling of the respiratory chain in HFE mitochondria, are paramount in the elucidation of a mechanism for the phenotypic expression of FE. Ojano-Dirain et al. (2007) found lower respiratory chain coupling, but equal to or greater oxidative phosphorylation capability in LFE broilers compared to the HFE. They also noted three mitochondrial proteins (COX II, cyt *c*1, cyt *c* and vinculin, a focal adhesion regulator) differently expressed with the FE phenotype whereas

Cox IV and ATPase α were implicated as greater in HFE compared to LFE. These findings are clearly consistent with our findings as presented in Tables 4-4 and 4-5, linking muscle type and FE to CxI 30, Cox II, Cox IV, ATPase α and CxII 30. In the shotgun proteomics study with male Pedigree broilers (PedM), Kong, et al. (2016) identified 228 mitochondrial proteins indicating a higher mitochondrial expression in the HFE phenotype, mitochondrial dysfunction and oxidative phosphorylation at 1 and 5 canonical pathways; predicted insulin receptor activated in HFE phenotype.

Total weight gain in the pigs selected for this study was not the controlling factor since no significant difference in total weight gain (TG) was observed. Total feed intake (TFI) was, however, remarkably different between the 2 FE groups, unlike the HFE and LFE broilers in the Bottje et al. (2002) study, where feed intake did not vary between phenotypic FE groups. In that study, they reported respiratory chain coupling was lower in LFE mitochondria whereas the generation of ROS was higher. Increased generation of ROS has also been associated with fast growth in pigs that have an associated high feed intake (FI). We have suggested that fast growth be specifically defined to; include growth of lean muscle (i.e., LM perhaps at the expense of SpD).

In the metabolic process, subcellular free radicals are produced by the electron transport chain in the mitochondria (Ames et al. 1993; Drogue, 2002). Mitochondrial DNA, which is relatively unprotected, may be vulnerable to oxidative stress (Wei, 1998). The 2-5% oxygen which is not reduced to water is converted to superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Mitochondrial DNA, relatively unprotected, and other biomolecular molecules are at risk for damage. The protein subunits of the electron transport chain (ETC) are especially important for proper functioning of mitochondria according to Nijtmans et al. (2002). ROS precipitated

deletions can lead to mistranslations in proteins (Wei, 1998). These faulty proteins are tagged for cell death. As a result, mitochondria can be destabilized, releasing critical stabilizing proteins to the cytoplasm. If cytochrome *c*, smac Diablo, or AIF is released, mitochondrial apoptosis with cellular decay occurs. This process leads to the preparation of the defected cell for phagocytosis. Iqbal et al. (2002 a and b) reported ubiquitin, which tags proteins destined to be degraded by proteasomes (Mehlhase and Grune, 2002), was higher in LFE than HFE broilers, suggesting a higher protein turnover in LFE birds.

In this study, we have focused on protein expression, proceeding from the general to the specific. First, we observed banding pattern differences in muscle types and interactions between muscle types and FE groups at band 7. The higher number of proteins expressed in porcine LM mitochondria in the LFE group supports findings that an accumulation of proteins in LFE broiler may be a compensatory response to oxidative stress (Iqbal et al., 2004) as in broiler breast meat. In our swine mitochondria, a higher expression of proteins in LM in HFE pigs and a lower expression of proteins in SpD in the LFE group supports Ruusunen and Puolanne (2004) suggestion of muscle type transformation in favor of light over dark muscle in HFE swine compared with LFE swine.

We observed a higher expression of protein carbonyls in the SpD compared to LM. However, Koo-NG et al. (2000), predicted a higher expression in the LFE mitochondria in the LM compared to the SpD muscle, suggesting muscle dependent amino acid side group specificity and lower capacity for light muscle to protect against exposure to oxygen consumption. They explained that type II fibers, abundant in light muscle, are expected to incur more damage by reactive oxygen species due to the lower concentration of several enzyme antioxidants in type II compared with type I fibers. However, in that study with mice exposed to

high levels of the hydroxyl radical after a six-week exposure to ethanol, they found that the capacity of type I muscle to resist free radical induced protein damage was significantly lowered. Just as continued exposure to ROS lowered the capacity of the red muscle to protect against protein damage, a trade off to HFE could be indicated in a higher production of and continued protein exposure to ROS. Therefore, expression of protein carbonyls in skeletal muscle and FE group mitochondria may be reflected at the complex or subunit level of respiratory chain.

A higher protein expression of respiratory chain subunits in LM compared to SpD lends support to the suggestion of compensatory expression of proteins for lower complex activities (Iqbal et al., 2003). Cx I and II activities were greater in the LFE group in the SpD compared to the HFE group but higher in the HFE group compared to the LFE group in the LM. This indicates a possible defect in the Cx I 30 subunit that may account for the differences in complex activities. If there is indeed a defect at this level in Cx I, destabilization of the mitochondria and loss of cytochrome *c* could precipitate mitochondrial apoptosis. In this case, activity levels of subsequent complexes and subunits in the electron transport chain (ETC) would be negatively impacted.

Iqbal et al. (2004) reported no differences in expression of ATPase subunits between HFE and LFE broiler breast mitochondria. Our findings with a significant difference between ATPase α expression between muscle types as well as FE groups in swine, suggest mitochondrial ATPase α expression, may be both muscle type and species specific. Since the expression of alpha ATPase was greater in both LM compared with SpD and HFE vs LFE mitochondria, we may deduct that alpha ATPase subunit is more highly expressed in HFE efficient pigs because of increased light fiber types associated with HFE swine.

Studies with other tissue may also support the phenotypic expression of FE via

mitochondrial protein Cx subunits. A study with heart muscle from male broilers by Tinsley et al. (2009) found the expression of 6 ETC proteins that were higher in lower FE mitochondria compared with high FE samples. In addition, one subunit of complex 1 was more highly expressed in HFE than in LFE mitochondria. Nara et al. (2018), with their blood analyses from efficient and inefficient heifer calves, inferred that high feed efficiency was associated with elevated potassium and phosphorus but reduced alkaline phosphatase. Muscle proteome data from studies with rats (Kim et al., 2011) showed that of 658 protein spots, 23 were differentially expressed for obesity prone (OP) vs obesity resistant (OR). They concluded lipid metabolism with muscle contraction and increased expression of marker protein for oxidative type I muscle contributed to OR, not antioxidative proteins.

Conclusion

The overall results of this study suggest a muscle type link between feed intake, protein oxidation, and protein expression in Cx I 30, Cx II 30, Cox II, Cox IV, and ATPase. Since feed intake was significantly higher in LFE compared to HFE, the added factor of calorie intake may have influenced the expression of carbonyl in SPD from LFE. Increased expression of the protein subunits may have been a compensatory response to carbonyls and the negative effect on respiratory chain.

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Tables and Figures

Table 4-1. Growth performance data for HFE and LFE swine within the same genetic line

	TFI (Kg)	ADFI (Kg)	TG (Kg)	FCR, (kg I/ kg G)	FE, (kg G:kg I)
HFE	81.89	0.78	47.51	1.72	0.48
	65.30	0.62	34.74	1.88	0.58
	89.17	0.85	46.74	1.91	0.52
	88.13	0.84	44.16	2.00	0.49
	97.04	0.93	47.86	2.03	0.48
	87.37	0.83	42.01	2.08	0.50
	107.65	1.03	51.13	2.11	0.53
LFE	110.38	1.05	39.92	2.77	0.34
	138.11	1.32	48.65	2.84	0.31
	113.74	1.09	39.03	2.91	0.34
	127.31	1.21	43.03	2.96	0.34
	116.64	1.11	39.25	2.97	0.35
	164.59	1.57	53.21	3.09	0.32
	126.48	1.21	39.40	3.21	0.36
HFE (mean)	88.08	0.84	44.88	1.96	0.51
LFE (mean)	128.76	1.22	43.21	2.96	0.34
P value; n=14	0.0005	0.0005	0.58	<0.0001	<0.0001
Growth period in days = 105					

HFE—high feed efficient = top 1/6 in feed efficiency from 427 Monsanto Choice Genetics pigs;
 LFE—low feed efficient = bottom 1/6 in feed efficiency from 427 Monsanto Choice Genetics
 pigs; TFI—total feed intake; ADFI—average daily feed intake; TG—total wt. gain; FCR—feed
 conversion ratio (kg intake:kg wt. gain); FE—feed efficiency (kg of wt. gain:kg of feed intake)

Table 4-2. Banding pattern main effect means (pixel intensity) for mitochondrial protein from HFE and LFE swine within the same genetic line

	Muscle		FE Group		P values; n=14		
	LM	SpD	HFE	LFE	SE	MP	IP
Band							
1	121.39	69.32	92.41	98.31	7.73	0.01	
2	68.33	35.85	47.78	56.40	4.77	0.01	
3	63.74	38.03	49.03	52.74	4.41	0.01	
4	44.61	25.84	33.68	36.76	2.61	0.01	
5	45.92	26.44	34.06	38.30	2.30	0.01	
6	64.65	27.27	44.82	47.10	3.44	0.01	
7	35.70	31.58	32.51	34.77	1.39	0.05	0.07
8	44.82	30.82	38.88	36.77	2.40	< 0.01	
9	44.57	29.71	39.18	35.10	2.78	< 0.01	
10	35.69	36.04	36.11	35.62	1.42	< 0.01	
11	56.25	31.86	43.93	44.18	3.86	< 0.01	
12	39.30	28.49	32.81	34.98	1.87	< 0.01	
13	45.27	29.21	35.23	39.25	2.31	< 0.01	
14	35.84	27.96	31.21	32.59	1.42	< 0.01	
15	31.40	30.83	31.55	30.68	1.52	< 0.01	
16	51.47	44.15	48.51	47.11	2.58	0.06	
17	32.23	28.48	29.63	31.08	1.78	< 0.15	
18	30.90	27.92	29.29	29.52	1.31	0.12	

Effects with P values ≤ 0.07 listed; all others are > 0.07 .

LM--*Longissimus muscle*; *SpD*—*Spinalis dorsi muscle*; 18 bands detected by 1D gel electrophoresis and Coomassie blue staining of mitochondrial protein (Figure 4-3). HFE—high feed efficiency = top 1/6 in feed efficiency of 427 pigs; LFE—low feed efficiency = bottom 1/6 in feed efficiency of 427 pigs; MP—muscle P value; IP—muscle * group interaction P value.

Table 4-3. Eleven subunits of the electron transport chain selected for immunoblotting assays

Complex	Protein Subunit	Mw (KDa)	Nomenclature	Alternative Name
Cx I	Cx I 15	12.5	NDUFA6	Iron-sulfur protein , 5
Cx I	Cx I 17	15.5	NDUFB6	B-complex, 6
Cx I	Cx I 30	30.2	NDUFS3	Iron-sulfur protein, 3
Cx I	Cx I 39	42.5	NDUFA9	A-complex, 9
Cx II	Cx II 30	31.6	SDHB	Iron-sulfur protein
Cx III	Core I	51.6	UCQRC1	None
Cx III	FeS	29.6	UCQRF51	Rieske iron-sulfur protein
Cx IV	Cox II	25.6	SCO2	COX II
Cx IV	Cox IV	19.6	Cox4	COX IV
Cx V	α	55.1	ATPA5A1	F ₁ complex, α subunit
Cx V	β	51.6	ATP5B	F ₁ complex, β subunit

Nicholls et al. (2013)

CX I to Cx V—Complex I to Complex V of the electron transport chain

Cx I 15—a subunit of complex I with a molecular weight of 12.5 KDa

Cx I 17 or CI17—a subunit of complex I with a molecular weight of 15.5 KDa

Cx I 30 or CI30—a subunit of Complex I with a molecular weight of 30.2 KDa

Cx II 30 or C230—a subunit of Complex II with a molecular weight of 31.6 KDa

Cor I—a subunit of complex III with a molecular weight of 51.6 KDa

FeS—a subunit of complex IV with a molecular weight of 29.6 KDa

CoxII or Cox2—a subunit of complex IV with a molecular weight of 25.6 Kda

Cox IV or Cox4—a subunit of complex IV with a molecular weight of 19.6 KDa

ATPase α —a subunit of complex V with a molecular weight of 55.1 KDa

ATPase β —a subunit of complex V with a molecular weight of 51.6 KDa

Table 4-4. Mitochondrial protein expression data (main effects means in pixel intensity) for muscle type and FE group from swine within the same genetic line.

Trait	Muscle		FE Group		P values			
	LM	SpD	HFE	LFE	SE	MP	GP	IP
Cx I 15	58.92	4.28	33.63	29.57	7.55	< 0.01		
Cx I 17	87.58	28.73	58.46	57.86	7.54	< 0.01		
Cx I 30	91.96	16.01	60.78	47.19	8.34	< 0.01		0.18
Cx II 30	137.22	24.76	73.36	88.63	9.01	< 0.01		
Cor I	91.93	15.77	56.77	50.93	8.97	< 0.01		
FeS	71.68	20.97	47.30	45.34	5.63	< 0.01		
Cox II	118.41	60.59	90.84	88.15	2.72	0.05		0.08
Cox IV	140.34	65.92	130.18	76.07	8.45	0.01	<0.01	0.18
ATPase α	109.93	74.65	104.42	80.17	6.99	0.01	0.02	
ATPase β	115.32	58.15	90.35	83.11	6.50	< 0.01		
Car I	116.70	125.93	116.82	125.81	5.21	< 0.01		0.04
Car II	121.16	139.01	129.06	131.12	3.77	< 0.01		0.02

Effects with P values \leq 0.20 listed: all other P values > 0.20.

LM—*Longissimus muscle*; SpD—*Spinalis dorsi muscle*; MP= P Values for muscle group.

IP = P Values for Interactions; GP=P values for FE groups.

HFE—high feed efficiency = top 1/6 in feed efficiency in feed efficiency of 427 pigs.

LFE—low feed efficiency = bottom 1/6 in feed efficiency of 427 pigs.

Cx I 15—a subunit of complex I at with a molecular weight of 12.5 KDa

Cx I 17—a subunit of complex I with a molecular weight of 15.5 KDa

Cx I 30—a subunit of Complex I with a molecular weight of 30.2 KDa

Cx II 30—a subunit of Complex II with a molecular weight of 31.6 KDa

Cor I—a subunit of complex III with a molecular weight of 51.6 KDa

FeS—a subunit of complex IV with a molecular weight of 29.6 KDa

Cox II—a subunit of complex IV with a molecular weight of 25.6 KDa

Cox IV—a subunit of complex IV with a molecular weight of 19.6 KDa

ATPase α —a subunit of complex V with a molecular weight of 55.1 KDa

ATPase β —a subunit of complex V with a molecular weight of 51.6 KDa

Car I—one of two carbonyl bands detected in porcine mitochondria via western immunoblotting

Car II—the second of two bands detected in porcine mitochondria via western immunoblotting

Table 4-5. Significant correlations among Cx subunits (pixel intensity) and FE¹ CMG

	CxI 30	CorI	FeS	CoxIV	ATPβ	CarI	FE				
CxI 15					0.60						
CxI 17	0.66	0.83	0.84								
CxI 30				0.73							
CorI			0.94								
FeS				0.60							
ATPα					0.68						
CoxII						-0.47*	0.68				
LM											
	CI17	CI30	C230	CorI	FeS	Cox2	Cox4	Aα	Aβ	CarII	FE
CxI 15	0.63	0.72	0.68	0.62	0.72	0.66	0.69	0.54	0.80		
CxI 17		0.86	0.62	0.92	0.93	0.74	0.67		0.53	-0.42	
CxI 30			0.76	0.96	0.96	0.71	0.83		0.49		
CxII 30				0.71	0.76	0.81	0.60		0.61	-0.38	
CorI					0.97	0.74	0.77		0.49	-0.44	
FeS						0.76	0.77		0.56	-0.40	
Cox2							0.64	0.50	0.75	-0.58	
Cox4								0.50	0.60	-0.38	0.50
ATPα									0.76	-0.41	0.41
ATPβ										-0.57	
CarI										0.76	
SpD											
	CxI30	CorI	FeS	CoxII	CoxIV	Aα	Aβ	CarI	CarII	FE	
CxI 15					0.71						
CxI 17	0.86	0.81	0.76								
CxI 30		0.77	0.73						0.49*		
CorI			0.59	-0.47*							
CxII 30					-0.58	-0.69				-0.66	
CoxII					0.59					0.58	
CoxIV						0.80	0.48*	-0.54	-0.45*		
ATPα								-0.52		0.78	
CarI									0.71	-0.56	
CarII										-0.53	

P < 0.05; * P < 0.10.

CxI 15—a subunit of complex I at with a molecular weight of 12.5; CxI 17—a subunit of complex I with a molecular weight of 15.5; CxI 30—a subunit of Complex I with a molecular weight of 30.2; CxII 30—a subunit of Complex II with a molecular weight of 31.6; Cor I—a

subunit of complex III with a molecular weight of 51.6; FeS—a subunit of complex IV with a molecular weight of 29.6; CoxII—a subunit of complex IV with a molecular weight of 25.6; CoxIV—a subunit of complex IV with a molecular weight of 19.6; ATP α —a subunit of complex V with a molecular weight of 55.1; ATP β —a subunit of complex V with a molecular weight of 51.6; CarI—one of two carbonyl bands detected in porcine mitochondria via western immunoblotting; CMG—combined muscle group—*Longissimus* and *Spinalis dorsi* combined values.

¹Note: values without * are significant.

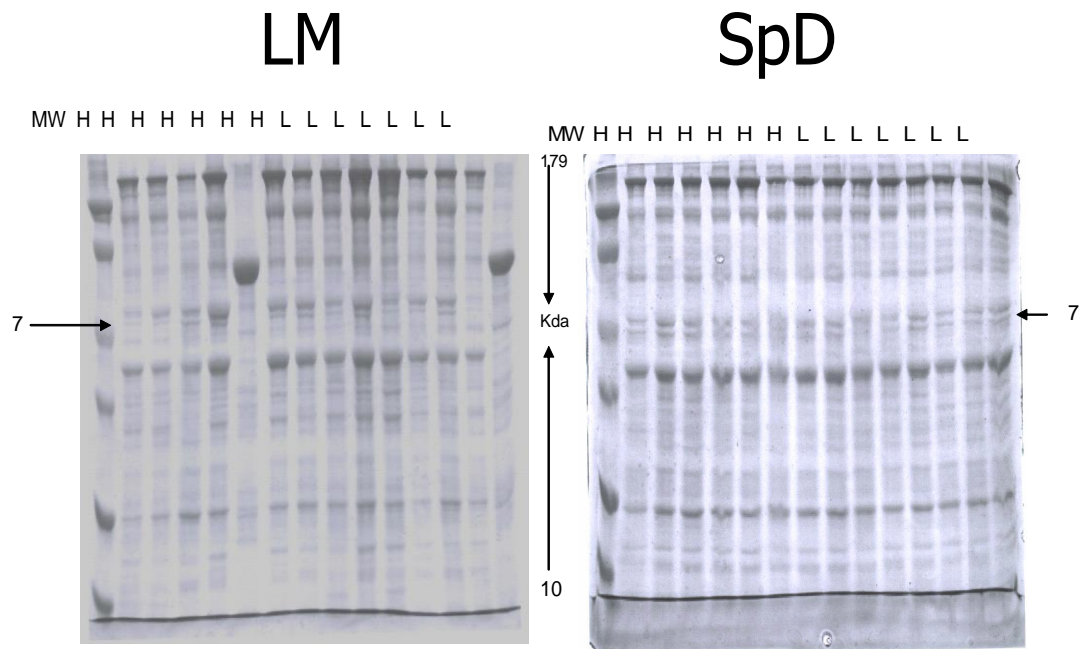


Figure 4-1. Banding pattern for mitochondrial protein in LM and SpD with one dimensional gel electrophoresis.

MW—molecular weight; H—HFE; L--LFE

LM—*Longissimus* muscle; SpD—*Spinalis dorsi* muscle

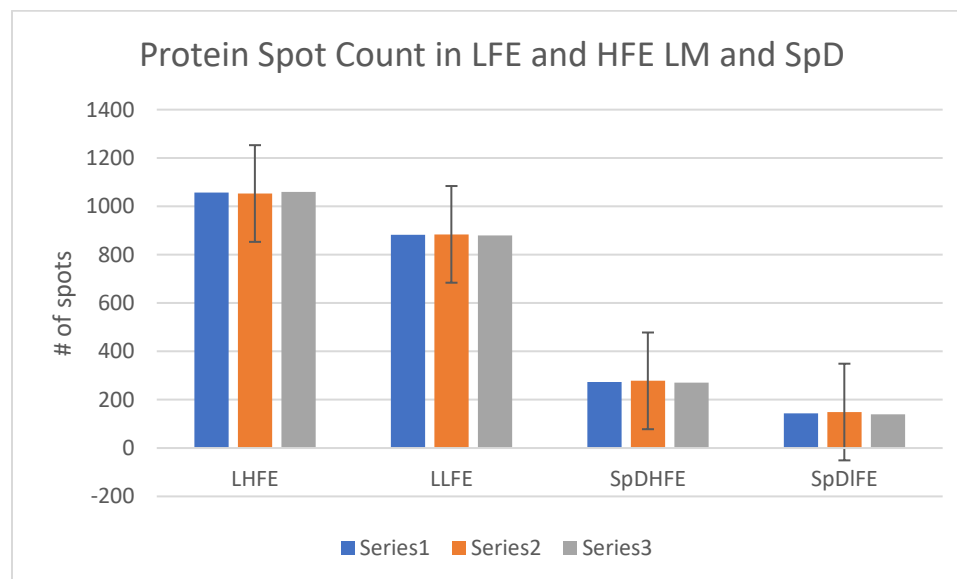
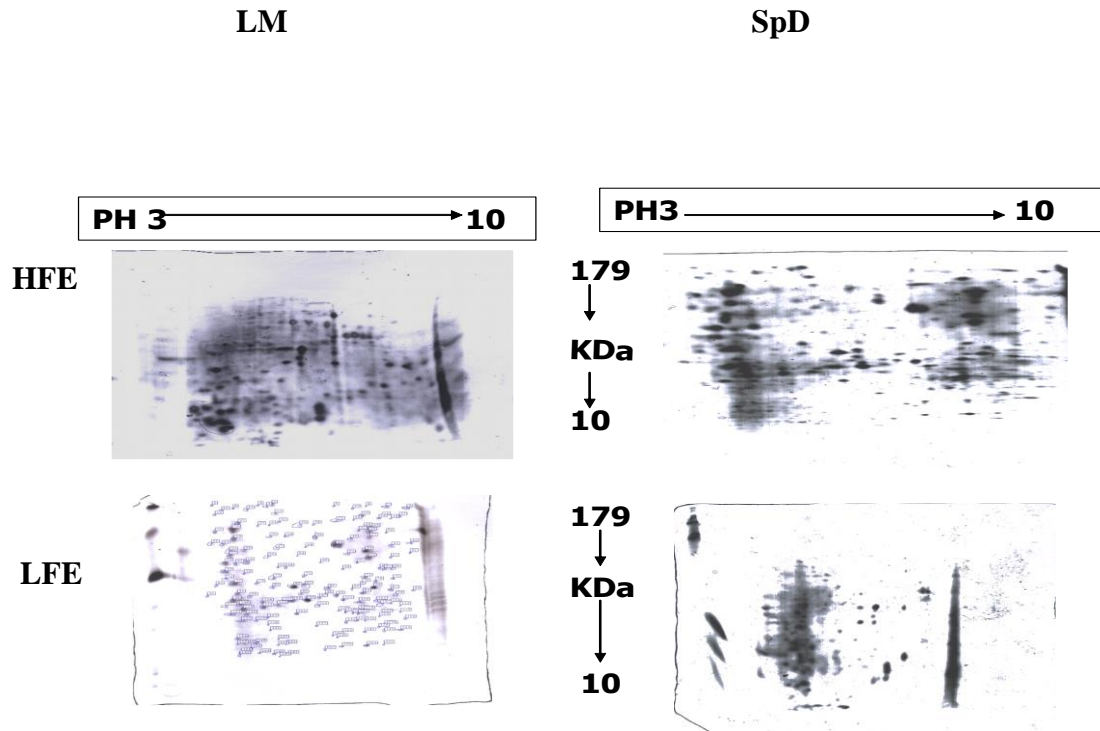
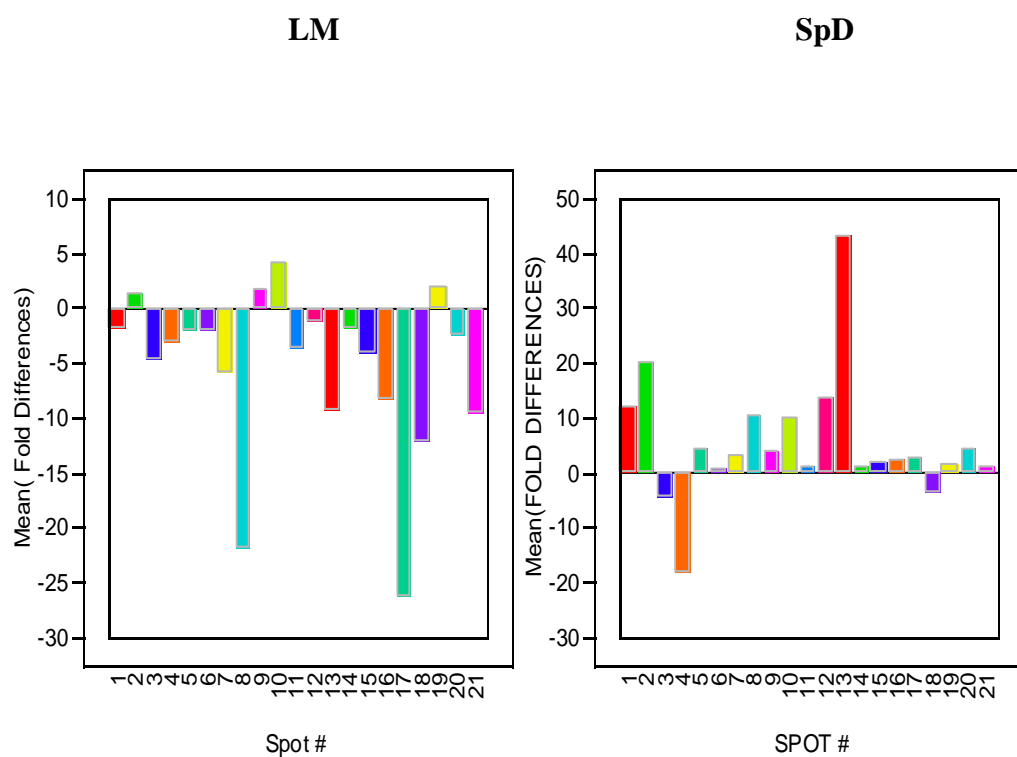


Figure 4-2. Banding pattern for mitochondrial protein in LM and SpD with 2-dimensional gel electrophoresis.
P < 0.05; n=3; a (LHFE), b (LLFE), c (SpD HFE), d (SpD LFE)--Least SQ means for number of protein spots.

LLFE =LM LFE; LHFE = LM HFE; SDHFE = SpD HFE; SDLFE = SpD LFE
a,b,c,d not alike indicate significant differences in protein spot counts.



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Spot #
130	128	114	115	120	110	110	105	120	105	53	55	60	60	90	80	59	50	50	39	35	MW
7.3	7.8	7.9	8.2	8.5	6.0	7.0	7.5	8.7	8.3	7.3	7.5	8.5	8.7	9.2	9.2	5.8	6.5	8.2	7.0	6.8	PH

Figure 4-3. LM and SpD mitochondria fold differences (pixel intensity) in protein spots from 2-dimensional gel electrophoresis.
P < 0.05; n=3

Positive values represent high feed efficiency pixel intensities; negative values represent low feed efficiency intensities.

Chapter 5

SUMMARY AND IMPLICATIONS FOR RESPIRATORY CHAIN ACTIVITIES AND PROTEIN EXPRESSION IN LONGISSIMUS AND SPINALIS DORSI FROM HIGH AND LOW FEED-EFFICIENCY SWINE WITHIN THE SAME GENETIC LINE

Conclusions

Feed efficiency (FE) remains the most important consideration in raising livestock to market value due to the cost of feed, which comprises 60-70 % of the total for marketable pigs. Therefore, researchers continue to investigate pathways that may lead to the elucidation of mechanisms for the phenotypic expression of Feed efficiency (FE). Current as well as past studies have provided evidence for a connection between macroeconomic/agricultural and cellular/biochemical basis of feed efficiency in farm animal production. Pioneers have linked FE to mitochondrial function in broilers, cattle, sheep, and other farm animals.

Our study with porcine mitochondria samples from high (HFE) and low (LFE) feed efficient pigs was a novel venture to determine if a link could be established between electron transport chain (ETC), muscle type and phenotypic expression of FE in swine. Therefore, we measured respiratory chain activities (Cx I to V) in Chapter 3 and protein expression (Chapter 4) in skeletal muscle mitochondria from HFE and LFE animals within same genetic line and fed the same diet. Ultimately, the intent was to determine if the impact of FE on mitochondrial function as established in broilers, and other species are similar in porcine models. We met our objectives and observed significant differences between muscle type as well as FE groups in mitochondrial enzyme activities and concluded that mitochondria are more efficient in LM compared to SpD

and HFE compared to LFE. Our study revealed a muscle*group interaction for Cx II. However, both Cx I and II activities differed significantly between swine muscle type as well as FE group with Cx I activity higher in HFE mitochondria compared with LFE in LM. Cx III to V activities were greater in LM compared to SpD. Complex II was higher in the LFE mitochondria than HFE in SpD. In the combined effects, Cx I and II activities were higher in SpD than LM, whereas activities for Cx III to V were higher in LM than SpD. In FE groups, Cx I activity was greater in HFE than LFE, whereas Cx II activity was higher in LFE, with most of that difference due to the higher activity in the SpD muscle. The general increase in activity from Cx I to III-V in HFE LM vs a remarkably high activity in Cx I and II and a decrease to very low levels in Cx III-V in LFE SpD indicates efficiency vs inefficiency of the ETC in LM and SpD, respectively. Several pathways have been cited to support compensatory responses to the respiratory activities and include but are not limited to apoptotic, mTOR (mammalian target of rapamycin), and myostatin links to FE as future projects for their association with ROS production, upregulation of protein, and muscle development, respectively.

In Chapter 4, in order to evaluate the difference in expressions of selected mitochondrial protein subunits, we compiled a list of immunoreactive proteins, which have been tested in other species and compared our findings. With UV spectrophotometry, one- and two- dimensional gel electrophoresis, we observed differentials in mitochondrial protein banding patterns, expression, and degree of protein oxidation between LM and SpD in HFE vs LFE swine skeletal muscles from pigs within the same genetic line and fed the same diet. Pixel intensity for ten protein subunits were higher in LM compared to SpD and two (Cox IV and ATPase α) greater in HFE than LFE porcine samples. Carbonyls (Car I and Car II) for Cox IV and ATPase α were higher in SpD compared to LM with significant associated interactions. Cox IV and ATPase α were

positively correlated to FE in LM whereas CxII 30 (responsible for muscle hypertrophy) was negatively correlated but Cox II and ATPase α were positively correlated to FE in SpD, which may have eliminated the normal increase in size of the skeletal muscle.

The overall results of this study suggest a muscle type link between feed intake, protein oxidation, and protein expression in Cx I 30, Cx II 30, Cox II, Cox IV, and ATPase. Since feed intake was significantly higher in LFE compared to HFE, the added factor of calorie intake may have influenced the expression of carbonyl in SpD from LFE animals. Increase in expression of the protein subunits may have been a compensatory response to carbonyls and the negative effect on respiratory chain activity.

Respiratory chain activity and fiber type in mitochondria from porcine skeletal muscle have been inextricably linked to the phenotypic expression of FE. Therefore, the objective of this study to examine the molecular basis of mitochondria isolated from *Longissimus* (LM) and *Spinalis dorsi* (SpD) muscle of high (HFE) and low (LFE) feed efficient swine and compare findings with existing reports has been met.

Implications

We look forward to validation studies targeting apoptotic, mTOR, and myostatin links to mitochondrial function and FE in farm animals. These findings not only impact the cost of growing farm animals to market value, but also provide approaches to medical applications. Mitochondrial function, for example, in insulin and obesity resistance may be associated with differences in feed or food intake as we observed herein. If red muscle is indeed disenfranchised due to enhanced metabolic rates and reactive oxygen species (ROS), we may include comparative antioxidants in the selected diet for lean growth as an anti-apoptotic ingredient.

Similar studies are developing in medical research in neurodegenerative diseases such as Parkinson's with muscle weakness and loss of balance. Dementia and Alzheimer's are also being investigated for links to mitochondrial efficiency. Therefore, our findings linking feed intake, respiratory chain activity, and protein expression to feed efficiency via mitochondrial function has also provided an avenue for continued research and validations that may extend to medical applications.

APPENDICES

APPENDIX I

Microplate Protein Assay

(Based on Sigma Kit #610-A, Sigma Chemicals)

1. Turn the Power WaveX on; go to KC junior and select the "Protein Assay".
2. Get Ready
Protein assay solution, Stds (300 mg/L), 0.85% NaCl and Control (if needed)
0.85% NaCl s, pipettes (1 mL, 100 (L), repeat pipette, tips, micro titer plate, lid, tubes etc.

Mix "Protein Assay Solution": 5 ml Protein Dye Reagent + 20 mL ddi H₂O.
3. Label tubes: 2 mL for each sample chose dilutions based on test (X30, X40, X50, X60, X70, X80)
1 mL for each Blanks, CNTL, Std. 1, 2, 3, 4
(1 mL for each sample (actual # or 1, 2, 3, 4))
4. Preparation of Control: (Urine Kontrolle, Level 1 #U9506)
Add the vol. Of ddi H₂O indicated on the vial label. Allow to stand for 5-10 min and then swirl gentle until dissolve completely. Do not shake, (good for 5 days at 2-8 °C).
5. Prepare dilutions: decide one dilution to be used for the type of tissue based on test runs.
X10 = Add 25 (L sample to 225 (L 0.85% NaCl
X20 = Add 25 (L sample to 475 (L 0.85% NaCl
X30 = Add 25 (L sample to 725 (L 0.85% NaCl
X40 = Add 25 (L sample to 975 (L 0.85% NaCl
X50 = Add 25 (L sample to 1225 (L 0.85% NaCl
X60 = Add 25 µL sample to 1475 µL 0.85% NaCl
X70 = Add 25 µL sample to 1725 µL 0.85% NaCl
X80 = Add 25 µL sample to 1975 µL 0.85% NaCl
X100 = Add 25 µL sample to 2475 µL 0.85% NaCl
X150 = Add 25 µL sample to 3725 µL 0.85% NaCl

•Prepare following for the final run:

	Solution (μL)	0.85% NaCl (μL)	Standard (μL)	Sample (μL)	Control (μL)	Conc (mg/mL)
Blank	500	100	-----	-----	-----	0
CNTL	500	-----	-----	-----	100	0.18-0.36
Std. 1	500	85	15	-----	-----	0.09
Std. 2	500	75	25	-----	-----	0.15
Std. 3	500	50	50	-----	-----	0.30
Std. 4	500	-----	100	-----	-----	0.60
SAMP1	500	-----	-----	100	-----	X30
SAMP2	500	-----	-----	100	-----	X40
SAMP3	500	-----	-----	100	-----	X50

- Mix thoroughly
- Incubate ~ 2 min at room temp
- To each designated well of microtiter plate transfer 100 μ L of BLK, CNTL, stds and Samples according to the “Microplate Plan Sheet”. **(Color is stable for at least 30 min).**

APPENDIX II

Citrate Synthase (Mitochondrial Marker)

The appearance of DTNB is proportional to the appearance or generation of CoA.

Reagents:

- 1 M Tris-HCl, pH 8.0
- 9 mM AcCoA (dissolved in H₂O)
- 1 mM DTNB (3.9 mg DTNB dissolved in 10 mL 1 M Tris-HCl buffer)
- 1% (v/v) Triton X-100
- 10 ug mitochondrial protein
- 9 mM Oxaloacetate (dissolved in 1 M Tris-HCl buffer)

Procedure:

1. Freeze-thaw mitochondria twice at -70°C to disrupt mitochondrial membranes
2. Set up the spectrophotometer at 412 nm (30 °C)
3. Choose the kinetic program: mix at 20 sec intervals
4. Run a + control to assess non-enzymatic hydrolysis
5. Sample assay:
 - Read absorption of reaction buffer (excluding OAA) for 3 minutes to measure AcCoA hydrolase activity or rate of non-specific AcCoA hydrolysis.
 - To initiate CS activity, 5ul OAA and obtain linear rates for at least 3 minutes

Total volume of the reaction is 220 ul.

Reagent	Positive Control	Sample
Tris Assay buffer	22 µL	22 µL
Triton X	22 µL	22 µL
DTNB	44 µL	44 µL
dH ₂ O	117 µL	117-sample
AcCoA	5 µL	5 µL
Sample	-	5 uL mito
Start the reaction by addition of 5 ul of 9 mM OAA and mix quickly		
Oxaloacetate	5 µL	5uL
Read results immediately; Absorption followed for 3 min		

Extinction coefficient = 13.6/umol

Ref

Zhang J. et al., 2002. in Mechanisms of Ageing and Development 123 (2002) 1363-1374.
Srere, P.A. 1969. Citrate synthase. Methods Enzymol. 13, 3-26

APPENDIX III

Microplate Complex Activity Assay

Complex I

(rotenone-sensitive NADH-Ubiquinone oxidoreductase activity)

Chemicals and reagents

1. **Complex I Buffer:** Tris-HCl, 1M, pH 8.0, (pH adjusted at 20~25):15.76 g Tris-HCl to 100 mL, adjusted to pH 8.0 with KOH, final volume to 90 ml + 0.5 g BSA.
2. NADPH, 15 mM: 11 mg NADPH dissolved into 1 mL ddi water.
3. 2,6-dichloroindophenol (DCIP), 1.3 mM: 19 mg DCIP to 50 mL.

Procedure

	Sample (μL)	Blank (μL)
Water	176 – mito	176
Mitochondria	5-10	---
Incubate in (37°C) chamber of spectrophotometer for 2 min temperature equilibration		
1Mtris-HCl	11	11
6Mm KCN	9	9
0.161mM AA	5	5
15mM NADH	11	11
1.3mM DCIP	8	8
Shake for 20s. Record the absorbance at 340nm for 3 min, v 6.22 mM⁻¹ cm⁻¹		
0.124mM Rotenone	7	7
Shake for 20s. Record the absorbance at 340nm for 3 min, v 6.22 mM⁻¹ cm⁻¹		

Option: Make Tris-HCl medium (supplemented with 5mg/mL BSA) plus the KCN and AA so that only one pipetting needed for these reagents.

APPENDIX IV

Complex II

(Succinate-Ubiquinone reductase)

Complex II medium, pH 7.4

- | | |
|--|-----------------------|
| 1. 55mM Potassium phosphate | (final conc. ~50mM) |
| 2. 0.1mM EDTA | (final conc. ~0.10mM) |
| 3. 20mM Sodium succinate | (final conc. ~18mM) |
| 4.65mM 2,6-dichloroindophenol | (final conc. ~mM) |
| Ubiquinone-2, 2.5 mM in ethanol | (final conc. ~mM) |

Procedure

Take out 1 microtiter plate

	<i>With Inhibitor</i>	
	Sample (μL)	Blank (μL)
Cx II Medium	183	183
Water	---	5
0.124mM ROT	7	7
Mitochondria suspension	5	---
Incubate at chamber of spectrophotometer for 3 minutes		
2.5mM Ubiquinone-2	7	7
DCIP	5	5
Mix (20s) and record the decrease in absorbance of DCIP at 600nm for 5 min , $v = 21\text{mM}^{-1}\text{cm}^{-1}$		
To measure isolate SDH activity, inhibit SQR with 1mM TTFA and add 0.8mM PMS as an electron acceptor which transfers electron from SDH directly to cyt c.		
TTFA	1mM	1mM
Mix (20s) and record the decrease in absorbance at 600mM for 5 min		

APPENDIX V

Cx II activity determined by the TTFA-sensitive rate (Cardoso et al., 1999)

Complex III (Ubiquinol-Cytochrome c Reductase)

Complex III medium, pH 7.8

1. 11.0mM Potassium phosphate
2. 5.5 mM magnesium chloride
3. 1.2mg/ml BSA
4. 2.0mM EDTA
5. 40.000mM KCN
6. 0.66mM Dodecyl maltoside
7. 0.220 ATP

Complex III is assayed at 550nm following the increase in absorbance resulting from the reduction of cyt *c* by ubiquinol-2. (Other ubiquinol analogues are less suitable.)

Preparation of Ubiquinol-2 (prepared fresh) Ref: Barrientos

1. To the decyubiquinone solution, add a few crystals of lithium/sodium borohydrate and pipette it up and down until the solution becomes transparent. Eliminate the excess of borohydrate with a few microliters of concentrated HCl, until no bubbles are produced. The final pH should be 2-3.

Reagent

Assay medium (final conc.): 10 mM Potassium phosphate, pH 7.8 at 20°C
2mM EDTA
5mM MgCl₂
1 mg/mL bovine serum albumin (fraction V)
240uM KCN

Get 10mL assay medium and add (final conc.):

4uM ROT
200uM ATP
0.6mM n-Dodecyl-β-D-maltoside

80uM Ubiquinol-2 in ethanol (final conc.): 2mg UQ2 in 2.5mL ethanol (see UQH2 prep. above).

40uM oxidized cytochrome c (III) dissolve in assay medium: 1mL medium + 0.0109g cyt *c*.

Reagents	Sample(mL)	Blank (mL)
Assay medium	195	198
Sample	3	---
0.88mM Oxidized cyt <i>c</i> (40um)	10	10

APPENDIX VI

Complex IV (Cytochrome c oxidase) assay (Barrientos, 2002)

This assay is performed at 550nm following the decrease in absorbance resulting from the oxidation of reduced cytochrome *c*.

Cx IV Potassium PO₄ Buffer, pH 6.8

	mw	conc	mL	wt(g)
KH ₂ PO ₄	136.1	11.0	0.030	0.045
Sucrose	342.3	275.0	0.03	2.824
BSA	na	1.2mg/mL	0.030	0.036

Reagents	Sample (mL)	Blank (mL)
Assay medium	202	205
Sample	3	-
Stir gently. Incubate 30s at 37 °C		
0.22mM reduced cyt <i>c</i> (10µM)	10	10
Mix (10s medium) and record absorbance for 2 min		
10.56mM KCN (240um final conc.)	5	5
Mix (10s) and record absorbance for 2 min		

APPENDIX VII

Complex V (ATPase) (Barrientos, 2002)

Complex V Medium, pH 8.0	g/20mL ddi H2)
50 mM Tris-HCl	0.158
50 mM KCl	0.075
MgCl ₂	0.081
5mg BSA 5mg/mL	0.108

Complex V activity can be measured spectrophotometrically by a coupled assay using lactate dehydrogenase and pyruvate kinase as the couple enzyme. The assay is performed at 340nm following the decrease in absorbance resulting from NADH reduction.

Procedure

1. In a microtube prepare a medium containing 200uL 50mM Tris(pH 8.0); 5mg/MIBSA; 20mMgCl₂; 50mM KgCl₂; 50mMKCl; 15uM carbonyl cyanide m-chlorophenylhdrazone; 5uM antimycin A; 10mM phosphoenolpyruvate (PEP); 2.5mM ATP; 4 units of lactate dehydrogenase and pyruvate kinase; and 1mM NADH. The addition of 3uM oligomycin allows for distinguishing the ATPase activity coupled to the respiratory chain. The decrease in absorbance is due to oxidation of NADH at 340 nm ($\epsilon = 6.81 \text{ mM}^{-1}\text{cm}^{-1}$).
2. Incubate the medium 5 min at 37 C.
Incubate mitochondria (20-40 ug of protein) in distilled water for 30s at 37 C.
3. Add medium to chamber to start the reaction. Follow the reaction for 3 min.
4. Add 3uM oligomycin and follow the reaction for an additional 3 min to distinguish the ATPase activity coupled to the respiratory chain.
Note: 1. PEP and NADH must be freshly prepared. 2. The expected inhibition with oligomycin is 60-90%.
5. Add following to medium:
15 Um CCP (carbonyl m-chlorophenylhydrazone cyanide)
5 uM Antimycin A
10 mM Antimycin A
10 mM PEP (phosphoenolpyruvate) (fresh)
2.5 mM ATP
20 U LDH (lactate dehydrogenase) and PK (pyruvate kinase)
1 mM NADH (fresh)
6. Assay: in microplate add 10 uL mitochondria + 43 uL medium + 162 uL di H₂O, wait 30s, read absorbance, 3 uM oligomycin read absorbance again

APPENDIX VIII

Reagents for Protein Expression Assays

TRIS BUFFERS:	1.50 M	91 g Tris 2 g SDS (sodium dodecyl sulfate); 0.4 % <i>q.s. to 0.5 L, adjust pH to 8.7 with HCl</i>
	0.25 M	15 g Tris 1 g SDS (sodium dodecyl sulfate); 0.2 % <i>q.s. to 0.5 L, adjust pH to 6.8 with HCl</i>

SAMPLE BUFFER (2-week shelf life):

5 mL	Tris Buffer, pH 6.8	0.125 M
1g	SDS (sodium dodecyl sulfate)	0.17 M or 5% (w/v)
4 mL	Glycerol	20 % (v/v)
2 mg	Bromophenol blue	0.02 % (w/v)
1 M	H ₂ O	
Add 12 mg DTT/mL before use (40 mM)		

RUNNING BUFFER (reusable ≤ 3 times):

12.1 g	Tris	0.025 M
57.6 g	Glycine	0.20 M
4.0 g	SDS (sodium dodecyl sulfate)	3.5 mM or 0.1 % (w/v)
<i>q.s. to 4 L, pH ~8.3 with HCl</i>		

TRANSFER BUFFER (reusable ≤ 3 times):

7.6 g	Tris	0.015 M
36 g	Glycine	0.12 M
800 mL	Methanol	20 % (v/v)
<i>q.s. to 4 L, pH ~8.3 Do not adjust!</i>		

OVERLAY BUFFER pH 8.7:

1.5 M	Tris	15 mL
0.4 %	SDS	0.24 g
Water		45 mL

Total	60 mL
-------	-------

BLOCKING BUFFER:

12.5 g Casein	2.5%
4.5 g Sodium Chloride	0.15 M
605 mg Tris	0.010 M
100 mg Sodium ethylmercutithiosalicylate (Thimersal)	0.02%
<i>q.s. to 0.5 L, adjust pH to 7.6 with NaOH</i>	

WASHING BUFFER (a.k.a. 0.5% Casein):

20.0 g Casein	0.5 % (w/v)
36.0 g Sodium Chloride	0.15 M
4.84 g Tris	0.010 M
0.80 g Sodium ethylmercutithiosalicylate (Thimersal)	0.02 %
<i>q.s. to 0.5 L, adjust pH to 7.6 with HCl</i>	

DETERGENT BUFFER:

200 ml Washing Buffer	
1 mL 20% SDS-solution	3.5 or 0.1% (w/v)
1 mL Triton X-100	0.5 (v/v)

20% SPS:

20 g SPS
100 mL ddi H₂O

TRIS SALINE:

12.1 g Tris	0.05 M
23.4 g Sodium Chloride	0.20 M
<i>q.s. to 0.5 L, adjust pH to 7.6 with HCl</i>	

GEL STAIN (reusable):

15 mL Glacial acetic acid	1.25 M or 7 % (v/v)
100 mL Methanol	~50 % (v/v)
100 mL Water	
0.40 mg Coomassie Blue R-250	0.2% (w/v)

GEL DESTAIN:

50 mL	Glacial acetic acid	0.8 M or 5 % (v/v)
450 mL	Methanol	~50 % (v/v)
500 mL	Water	

Tris 0.5 M pH 6.8
6.07 g → 100 mL Ddi water

Sample buffer (2X)

5 mL Tris buffer pH 6.8 (0.5 M)
1 g SDS
4 mL glycerol
2 mg Bromophenol
1 mL H₂O
(Add 12 mg DTT)

Tissue Homogenate buffer: Adjust pH with KOH just prior to use.

	w/EGTA
	pH 7.4
(Schnaitman & Pedersen, 1968)	<u>1 liter</u>
220 mM D-Mannitol (FW=182.2)	40.1 g
70 mM Sucrose (FW=342.3)	24.0 g
2 mM HEPES (FW=238.3)	0.5 g
1 mM EGTA (FW=380.4)	0.4 g
d H ₂ O	~1 liter
<hr/>	
292mOsM	

APPENDIX IX

Western blot

MINI GELS

Preparation of 10 mini gels:

		1D		2D		
7% T (mL)	7.5% T (mL)	8.% T (mL)	9% (mL)	10% (mL)	12% (mL)	
21	16.9	24	27	22.5	27	Protogel™ (40% Acrylamide 0.8% Bis-Acrylamide)
22.5	22.5	22.5	22.5	22.5	22.5	1.5 M Tris-HCl Buffer, pH 8.7
46.5	50.6	43.5	40.5	45	40.5	Distilled water

- Mix these ingredients and degas for ≥ 15 min.
 - Use this time to put together the glass plates and load them into the casting stand.
- Add and mix for 10 seconds
 - 500 μ L 10% (w/v) Ammonium persulfate (AMPS) (made up fresh)
 - 50 μ L TEMED
- Pour into the casting-stand immediately, start pouring in the middle up to the upper part of the black line (2-3) mm below the comb teethes).
- Then carefully add an overlay of ≤ 0.5 mL water-saturated n-butanol to each individual gel, get the top layer.
- After the gels have polymerized (≥ 1.5 hr) wash away the butanol and replace it with a layer of over-lay buffer.
- Then disassemble the unit by using edge of a razor, remove wax paper
 - Store (≤ 1 month) gels individually wrapped in saranwrap at 4 °C.

Prepare stacking gel (3% T) 1-2 hr prior to loading of the samples. Recipe for stacking gel for 4 minis:

1.5 mL Protogel™ (Acrylamide-Bis, 40%)
 10 mL 0.25 M Tris-HCl buffer, pH 6.8 w/v / 0.2% SDS
 8.5 mL Distilled water

- Combine these ingredients, degas for ≥ 30 min, and stir continually, use hood.
 - Meanwhile, pour off the overlay buffer from the running gels and dry the gel surface with filter paper (or paper towels) and fix it in the running apparatus with the help of clamps (one side small and other large).
 - Mark the top of the separating gel with a Lab Marker.

- Put the comb (1mm) in the stacking gel area, there should be some space left between gel mark and the comb.
- When degassing is complete, add the following to the mixture:

260 μ L 10% (w / v) Ammonium persulfate (AMPS) (made up fresh- **good for 3 days**) (1mg/10 μ L is 10%, Take 0.02 to 0.04 g or 20 or 40 mg and add the 10X μ L of ddi water)
26 μ L TEMED

Note: the quantities mentioned above are not absolutes; they may be change keeping in view how the mixture is congealing, normally wait 3-5 min.

- Mix, and pour into the casting-stand immediately.
- Slide an appropriate comb between the plates and fill each unit to the top and.
 - Do not trap air bubbles under the teeth of the comb(s).
 - Use a marker pen to indicate on the glass the position of individual wells (or **use a template**).
- After 1-1.5 hr carefully take out the combs by holding the comb in the middle while securing the casting in both hands.
- Rinse the wells 3X with running-buffer.
- Finally, fill the wells with running buffer and load your samples (the marks on the glass should assist you to load the wells properly).
 - Use fine longer tip to load the samples
 - Press little more the pipettes that will help avoid any bubbles
 - Try to be little below the level of the sample

Western blot

Sample Preparation:

Things Needed:

- Samples
- Sample Buffer and dithiothreitol (DTT)
 - Sample are prepared at the very least a 1:1 mixture of protein sample and sample buffer.
 - Just prior to use dissolve 6 mg dithiothreitol per ml of sample buffer.
- Standard (biorad) high or other one where the different color bands can be seen while running.
- Running buffer

Loading of the gels:

- Place all samples (including standards; see below) for 3 min in a 100 °C water bath and vortex-mix before loading them onto the gel.
 - When applying a mixture of proteins (e.g. microsomes) the total amount of protein loaded commonly ranges anywhere from **10** (for Western Blotting with Rabbit antisera or Coomassie stain) to **150 μ g/lane** (for Western Blotting with Human antisera).

- If a purified protein is loaded on the gel use about a 100-fold less protein (i.e. 0.1-1.5 µg / lane). Sample buffer is stored at room temperature and made fresh every 2-3 weeks.
- When using a 10-well comb the maximum load volume per well is $\pm 40 \mu\text{L}$.
- Avoid delays between loading of the sample and connecting the power source.
- On each gel at least one lane is usually reserved for **molecular weight markers**.
 - Use a standard protein mixture from Biorad, either “**low range**” (up to 96kDa) and / or “**high range**” (up to 200 kDa).
 - Good results are obtained by loading 10µL / lane of mixture containing 2 µL standard, 50 µL sample buffer.
- Any left-over samples that are mixed with sample buffer are usually discarded.
 - Precious samples, however, can be successfully reused if they are promptly frozen at -20 °C; to reuse such a sample just heat it for 3 min at 100°C, mix, and you are ready to load.

Running of the gels:

- Fill up the space behind each gel with running buffer, all the way to the top of the unit. Use the constant-voltage mode of the power supply.
 - Stack: 100 V (takes 15-30 min or all the dye passes stacking)
 - Separation: 200 V (takes 1-2hr)

(Note: During stacking expect a current (i) of ~35 mAmps / 2 gels, during separation i ~ 65-25 mA / 2 gels)

Western blot

Note: In electrophoresis we run basically on Volts, move mAmps way up so that cannot interfere with the running.

“**Stack**” refers to migration of the dye from the well in which it was loaded to the top of the separating gel (which you marked on the glass).

“**Separation**” describes dye migration through the 7% T part of the gel.

- Turn off the power source as soon as the dye front has reached the bottom of the gel.
 - At this point proceed, **without delay**, to either
 - Stain the gel with Coomassie for a no-specific detection of protein*,
 - Or if immunochemical detection of one (type of) protein is desired transfer the proteins onto nitrocellulose paper or PVDF (better as it holds the protein better).
 - See the appropriate sections entitled COOMASSIE STAIN, and WESTERN BLOTTING, respectively.

WESTERN BLOTTING

AIM: To immunochemically detect 1 specific protein after SDS-PAGE

Blotting of electrophoretic protein(s) onto nitrocellulose (NC) or PVDF:

Things Needed:

Transfer buffer

Blotting apparatus including electrode head

Filter Paper

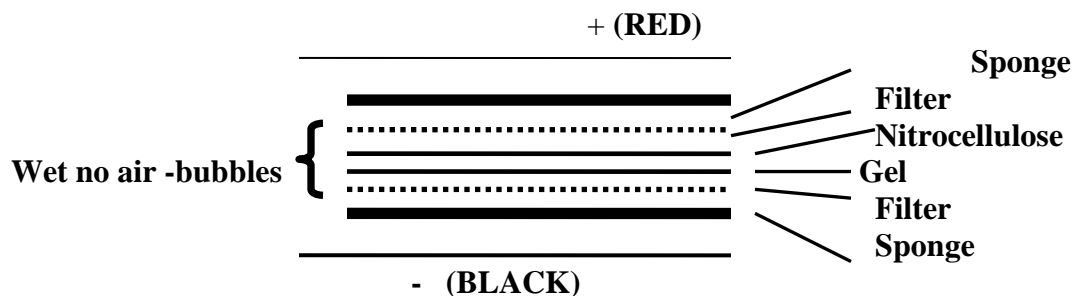
Nitrocellulose paper or PVDF

Tray

Razor blade

Flat forceps

- While the electrophoresis is still in progress cut (same dimensions as the gel) 2 pieces of *filter paper* (3MM Chromatography paper, comes in 17" x 23" sheets) and 1 piece of *nitrocellulose paper* or PVDF for each gel.
- Pour some *transfer buffer* in a plastic tray to soak the filter- and nitrocellulose paper for **10-15 min.**
- In the plastic holder place first a (dry) sponge, and on top of this a wetted piece of filter paper.
- Disassemble the gel electrophoresis unit and, with the gel facing you, remove the top glass plate. Separate and discard the stacking gel and cut off the **lower left-hand** corner of the gel.
- Pick up the gel (use gloves) by the corners and gently put it face-down on the wetted filter paper.
- On top of the gel lay down a piece of wetted nitrocellulose paper, followed by wetted filter paper.
 - Prevent air-bubbles from getting stuck between any of the layers by rolling the glass tip of 5 mL pipette (3-4 rolls on NC and filter paper); keep everything very wet.
- Finish the stack with a dry sponge and close the plastic tray.
- Mount the stack in the tank filled with *transfer buffer* and connect the electrodes; connect the red pole (i.e., anode, **on front side**) to **the nitrocellulose-side** of the stack, see schematic
 - Black side of the anode **MUST** on back side
 - Protein transfer is from black to red side (back to front)



- Choose one of the following constant-voltage transfer conditions:
 - Mini gels: 1hr. @ 200V, or 2 hr. @ 100v
 - Large gels: 2 hr. @ 250 V (use water bath cooling plus external MeOH/ice)

or 3 hr. @ 200 V (use water bath cooling plus external ice bath)

- or overnight @ 100 V (water bath cooling is sufficient)

(2 hr. @ 200 V, or overnight @ 80 V and then 1 hr @ 100 V)

Note: Minis; i~? → ? mA

@ 250 V i~600 → 2100mA

@ 250 V i~470 → 1750 mA

@ 100 V i~300 → 500 mA

Thin gels 80 V overnight then 100 V 1hr

For quick 400 m Amps or 0.4 V for one hour.

- After completion of the transfer place the “sandwich” **down on the table** (black side of the holder up) with gel facing up.
- Open the unit and carefully peel away layers until you reach the gel; mark its position on the nitrocellulose with a dull point pencil or marker pen.
- Use an appropriate comb to mark the position of lanes that will be stained for protein detection (e.g. molecular weight standards) or that will be incubated with different primary antibodies.
 - Coomassie stain gel to **determine completeness** of the protein transfer.
- Cut off the lanes that will be **protein-stained** and handle them as described below.
- Lanes earmarked for immunochemical staining are placed in blocking buffer for 1-2 hrs. or overnight at 4 ° (shake on orbital shaker) and treated as described in the subsequent section. How long we can store it 4 wk or more than that.

Detection of bottled molecular weight markers

- Place nitrocellulose stripes in “Nitrocellulose Stain” for 5 min, shake well.
 - Protein stain can be reused.

APPENDIX X

COOMASSIE STAIN

AIM: To visualize all proteins in a sample after SDS-PAGE

Stain:

- Before carrying out the actual staining procedure it is optional to “fix” the gel in 25% (w / v) trichloroacetic acid (TCA) for 0.5-1hr. Make sure to rinse the gel water when fixing is completed.
- To stain the gel(s) bathe it in Coomassie staining solution and agitate (≥ 4 hr) on an orbital shaker.
 - Individual mini gels are put in the lid of a box of pipette-tips with ± 20 mL staining solution.
 - Large gels or groups of minis are put in a low Nalgene box with 50-70 mL solution.
 - When the staining is continued for a long period of time (e.g. overnight) the container should be sealed to avoid evaporation methanol (this is one of the ingredients of the Coomassie staining solution).

Note: The staining solution is reusable. When the gel is stained properly it has a dark blue appearance and protein bands are only visible over a lightbox.

Destain:

AIM: This process will remove background stain.

- Rinse the gel with distilled water to remove excess staining solution. Then submerge gel in Coomassie destain solution.
- Agitate on orbital shaker for ≥ 2 hr, before repeating the procedure (i.e. pour off old destain, rinse with water, and add fresh destain).
- This process is repeated 2-3 times until optimum contrast between protein bands and background has been achieved.
 - Alternatively, add a destain sponge to the tray; in this way destaining can be completed without any changes of the destain solution, but it takes overnight.
- Finally, rinse the gel with water, soak it for 1 hr and take a picture.
- The gel can now either be stored in water (semi-permanent solution, ≤ 3 weeks) or soak ≥ 1 hr in 10% acetic acid solution with 1% glycerol, to prepare for gel-drying (intended for permanent storage).

General immunoblotting procedure

- **1° antibodies:**
 - Aliquot: 200 μ g/1000 μ L (depending on Ab and recommendations)
 - 1: 500 -----8 μ L + 4 mL washing buffer (Ab dependent)
 - Seal in the plastic bags for **Overnight** and put it on orbital vortex. **Avoid bubbles.**

- Washings (see instruction under immuno detection of specific proteins)
 - 2° antibodies:
 - Rabbit antisera (peroxidase labeled), Dako
 - 1: 20,000= 1μL (2°ab) + 20 mL of washing buffer (good for One blot)
- Add **20 mL** and each membrane for **90 min** in tip lid.
 - (Washings (see instruction under immuno detection of specific proteins the folder))
- **Substrate:** Super signal west dura extended (Pierce) (A & B, 50:50).
 - Make 3 mL by adding 1.5 mL of each substrate (good for one blots)
 - Seal 3 mL of substrate in a plastic bag and let it wait for 5 min, make sure no bubbles in it.
 - Drain the substrate
 - Put the membrane in Reaction Folder
- **Chemiluminescence Reading:**
 - Put the reaction folder in the third shelf of Fuji image analyzer (**Note: turn the machine on during incubation or before incubation starts**).
- Auto run, save this as an “image file (.IMG)” in the folder “Iqbal” and import as “tiff” file on a 100-zip disk.
 - Name the file with the primary antibody’s name.

Comments:

Immunochemical detection of specific proteins

1. Drain blocking buffer and cut the blot in sections that will be incubated with the reagents.
2. Make an appropriate dilution of the primary antiserum in 0.5% casein (Washing buffer). Incubate the blot for 90 min at room temperature or overnight at 4 °C.
3. **Washing**
 - Drain 1° antiserum
 - Rinse 3 times with DI water
 - Incubate 5 min with “Detergent Buffer”
 - Drain and rinse 3 times with DI water
 - Incubate 5 min with Washing Buffer
 - Drain and rinse 3 times with DI water
 - Incubate 5 min with “Washing Buffer”
 - Drain and rinse 3 times with water
4. 2° Antibody
 - Incubate blot for 90 min at room temperature or overnight at 4° C in an appropriate dilution (typically 1:1000 to 1:3000) or 2°.

5. **Washing**

Drain 2° antiserum

Rinse 3 times with water

Incubate 5 min with “Detergent Buffer”

Drain and rinse 3 times with DI water

Incubate 5 min with “Washing Buffer”

Drain and rinse 3 times with water

Incubate 5 min with “Tris-Saline”

6. Development. Substrate for development is dictated by the enzyme attached to the 2° Antibody in step 4.

APPENDIX XI

Mini 2D Gel Electrophoresis

Reagents

Rehydration stock solution without IPG Buffer₁

(8 M urea, 2% CHAPS, bromophenol blue, 25 ml)

Chemical	Final concentration	Amount
Urea (FW 60.06)	8 M	12 g
CHAPS ₃	2% (w / v)	0.5g
Bromophenol blue	trace	(a few grains)
Double distilled H ₂ O		
Store in 3.0mL aliquots at -20 °C.		

DTT and IPG Buffer are added just prior to use. Add 8 mg DTT per 3 mL aliquot of rehydration stock solution. For IPGPhor 15 µL of IPG Buffer (3-10NL) just prior to use.

Note: Stock solution can be stored in 3 mL aliquots at -20 °C.

Stock SDS equilibration buffer₁

(50mM Tris-C1 pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 200 mL)

	Final Concentration	Amount
1.5 M Tris-C1, pH 8.8 See General Electrophoresis	50 mM	6.7 mL
Urea (FW 60.06)	6M	72.07 g
Glycerol (100% v/v)	30% (v/v)	60 mL pipette slowly
SDS (FW 288.38)	2% (w/v)	4.0g
Bromophenol blue	trace	(a few grains)
Millipore water		To 200 mL

Store in 30 ml aliquots at -20 °C.

₁ This is a stock solution. Prior to use DTT or Iodoacetamide is added

Running buffer:

2.1 g	Tris	0.025 M
57.6 g	Glycine	0.20 M
4.0 g	SDS (sodium dodecyl sulfate)	3.5 mM or 0.1% (w/v)
q.s. to 4 L, pH ~ 8.3. pH w/ HCl		

Agarose Sealing Solution

Final	Concentration	Amount
SDS Electrophoresis buffer (running buffer)		100 mL
Agarose	0.5%	0.5 g
Bromophenol blue	0.002%	trace

Add all ingredients into a 500 mL beaker. Heat with a heating stirrer until the agarose is completely dissolved. Do not allow the solution to boil over. Dispense 2 mL aliquots into screw-cap tubes and store at room temperature.

Methods for 2D gel electrophoresis

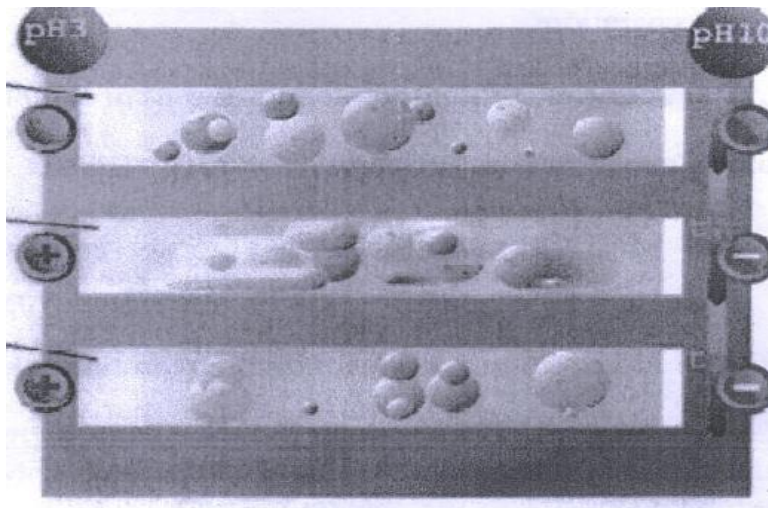
The method for Mini-2D gel electrophoresis is a composite from several sources.

2-D Electrophoresis Amersham Biosciences

DryStrip

IPGphor

First dimension is isoelectric focusing using Amersham Pharmacia Immobiline Dry Strips. Proteins will be focused on the Dry Strips using the IPGPhor by Amersham Pharmacia.



Immobilized DryStrip gels contains a pre-formed pH gradient immobilized in a homogeneous polyacrylamide gel. The gels are cast on a plastic backing and delivered dried. Prior to use, they are rehydrated in rehydration solution containing a matching IPG buffer. Package content: 12 Immobiline DryStrip gels and instructions.

Gel size: 68 x 3 x 0.5 mm

Storage: - 20 °C

Shelf Life: See expiry date on package

Anode: Arrow-pointed end

Note: To prevent contamination from skin keratin, always wear laboratory gloves when handling Immobiline DryStrip gels and all apparatus/solutions used in their preparation.

Rehydration of Immobiline DryStrip gels:

Immobiline DryStrip gels are rehydrated individually in the Ettan IPGphor Strip Holder that will be placed on the IPGphor and programmed as outlined below.

Working rehydration solution is made by adding IPG buffer just prior to use (Final concentration of 0.5% IPG Buffer and DTT in rehydration solution)

IPG Buffer must be the same pH interval as the Immobiline DryStrip gel that is being used at this time, we are using the 3-10 NL (nonlinear) Dry Strips.

Working Rehydration Solution:

15 µL IPG Buffer 3-10 NL in 3 ml of rehydration solution (see below) and

8 mg of dithiothreitol (DTT) in 3 mL of rehydration solution

Sample preparation:

Example: you want 25 µg of protein 125 µL of sample in working rehydration solution.

(Sample Concentration) * (Sample Volume) = (Final Concentration) * (Final Volume)

$$(13.33 \mu\text{g}/\mu\text{L}) * (X \mu\text{L}) = (25 \mu\text{g}/125 \mu\text{L}) * (300 \mu\text{L})$$

$$X \mu\text{L} = 4.5 \mu\text{L in } 295.5 \mu\text{L of working rehydration solution}$$

1. Pipette 125 µL of sample in working rehydration solution into an IPGphor Strip Holder.
2. Carefully, remove the plastic back by peeling the Immobiline DryStrip starting at the blunted end while holding with a flat tweezers. Label the blunt end with sample number using a-very-fine marker.
3. Carefully, place the Immobiline DryStrip in the tray channel, with the gel side down and the anode (arrow pointed end or + end) placed at the pointed end of the Strip Holder. Avoid trapping air bubbles under the strip. Line up all the tips of the gels to same spot in the Strip holder.
4. Slowly overlay the strip with PlusOne™ DryStrip Cover Fluid starting at the anode (pointed) end and working toward the cathode.
5. Rehydrate for 10 to 18 hours to make the running conditions for an overnight run as described below for the Ettan IPGphor.

Running conditions for the Ettan IPGphor:

The loading of the sample should be completed at 2.30 PM and samples removed at 9.00 AM the next day.

Use 50 µA per strip. The voltage program for first dimension

Isoelectric focusing is:

Step	Voltage	Duration	Current kVh	Voltage gradient type
Rehydration		16		
1	500	0:30	0.25	Step-n-hold
2	1000	0:30	0.5	Step-n-hold
3	5000	1:30	8.0	Step-n-hold

The loading of the sample should be completed at 2.30 PM and samples removed at 9.00 AM the next day.

Running conditions for the Ettan IPGphor:

The loading of the sample should be completed at 2.30 PM and samples removed at 9.00 AM the next day.

Use 50 μ A per strip. The voltage program for first dimension Isoelectric focusing is:

Step	Voltage	Duration	Current kVh	Voltage gradient type
Rehydration		16		
1	500	0:30	0.25	Step-n-hold
2	1000	0:30	0.5	Step-n-hold
3	5000	1:30	8.0	Step-n-hold

IPG strip equilibration

The equilibration step saturates the IPG strip with the SDS buffer system required for the second-dimension separation. The equilibration solution contains buffer, urea, glycerol, reductant (DTT), SDS, and dye. An additional equilibration step replaces the reductant with iodoacetamide. Iodoacetamide alkylates thiol groups on proteins, preventing their reoxidation during electrophoresis.

Equilibration Steps:

Note: The second-dimension vertical gel must be ready for use prior to IGP strip equilibration. See general electrophoresis method for preparation of Tall (10 cm) 10% T Vertical gels for the mighty small (Hoefer SE 260) respectively.

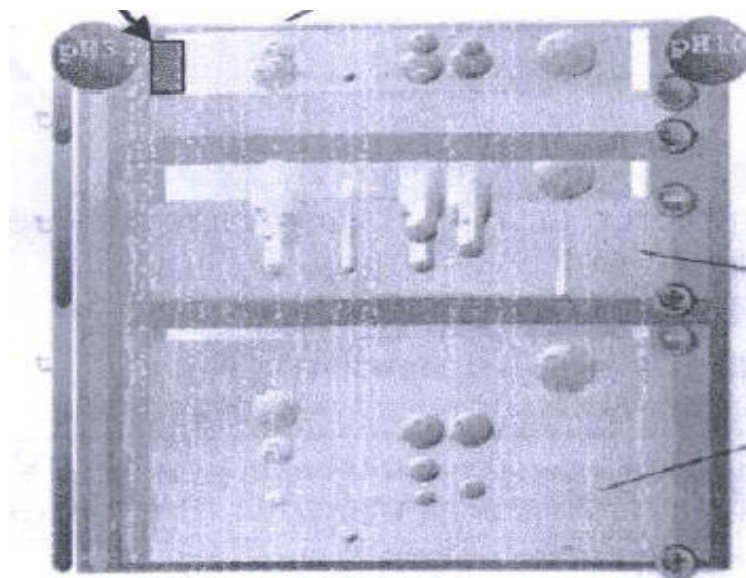
1. Prepare equilibration solution from frozen stock SDS Equilibration buffer (see below).
2. Split the 30 ml of stock equilibration buffer in half. Just prior to use, add **150 mg DTT** per 15 mL SDS equilibration buffer for the first equilibration.
3. Remove IPG strip from the Strip Holder and gently rinse with Millipore water (from a squirt bottle while holding blunt end with a tweezer). Then place the strip gently into a 10 mL polypropylene tube in individual tubes with the support film toward the wall. Add 2 mL of the DTT-containing solution to each tube. Cap the tube and place it on its side on a rocker. Equilibrate for 15 min.
4. A second equilibration may be performed with an iodoacetamide solution (without DTT). Prepare a solution of **375 mg iodoacetamide** per 15 mL stock SDS equilibration buffer.

Note: This second equilibration step reduces point streaking and other artifacts.

Pour out the DTT-containing equilibration solution and add 2 mL of an iodoacetamide equilibration per tube. Cap the tube, place it on its side on a rocker, and equilibrate for 15 min.

Tip: The mini-gel Hoefer SE260 electrophoresis unit should be set up and ready for the placement of the IPG strip and sealing solution should be warmed to 100 °C. The eight makers should be heated for 3 minutes at 100 °C.

2nd Dimensional Electrophoresis:



After the second equilibration remove the IPG strip and dip into running buffer to lubricate the gel. Position the IPG strip between the plates on the surface of the second-dimension gel in the Hoefer SE260 unit with the plastic backing against one of the inside glass plates. With a thin plastic ruler, gently push the IPG strip down so the entire lower edge of the IPG strip is in good contact with the surface of the slab gel (note it is important to have a level 2nd dimension gel). Ensure that no air bubbles are trapped between the IPG strip and the 2nd dimension gel.

Place a sample application piece (the original application piece should be cut in two prior to use) at the pointed end of the IPG strip (anode). Apply 5 μ L of the molecular weight (Sigma M0671) marker that has been diluted 1:10 (1 μ L in 9 μ L) in sample buffer containing DTT that has been heated at 100 °C for 3 minutes to the application piece. Pipette melted agarose sealing solution (let it cool slightly) to the top of the gel to seal the IPG strip and application piece.

APPENDIX XII

SilverQuest Silver Staining Solutions Protocol

(According to the Silver Quest Silver Staining Mass Spectrometry Compatible Kit by Invitrogen)

- A Total of 50 mL per mini slab gel (8-10 x 10 cm) is needed of each of the following solutions. Freshly made solutions (not older than around ~24h) give the best results.

<u>Step</u>	<u>Solutions</u>	<u>Amount</u>	<u>Time Exposure</u>
Fixation	Ethanol Acetic Acid (glacial)	100mL 25mL	20 min (or 1-2 days)
Sensitizing	Ethanol Sensitizer DdI Water	30mL 10mL 60mL	10 min
30% Ethanol Washing	DdI water Ethanol	70mL 30mL	10 min
Staining	Stainer DdI Water	1mL 99mL	15 min
Developing	Developer Developer enhancer Ddi Water	10mL 1 drop 90mL	2-5 min
Stopping ♦	Stopper	10mL	10 min

♦ The stopping solution has already been prepared.

Make sure to take a scan and save picture in the lab notebook.

APPENDIX XIII

Silver Staining Protocol for Proteins

(According to the Plus One Silver Staining Kit by Amersham Biosciences)

- A Total of 62.5 mL per mini slab gel (8-10 x 10 cm) is needed of each of the following solutions. Freshly made solutions (not older than around -24h) give the best results.

Step	Solutions	Amount	Time of Exposure
Fixation	Ethanol Acetic Acid (glacial) Ddi Waters	100mL 25mL 125mL	30 min
Sensitizing	Ethanol Glutardialdehyde (25% w/v) Sodium Thiosulphate (5% w/v) Sodium acetate	75mL 1.25mL 10mL 17g	30 min
Washing	ddI water	~	3x5 min
Silver Reaction	Silver Nitrate (2.5% w/v) Formaldehyde (37% w/v)●●	25mL 100μL	20 min
Washing	ddI	~	2 x 1 min
Developing	Sodium Carbonate Formaldehyde 37% w/v)●●	6.25g 50μL	2-5 min
Stopping	EDTA-Na ₂ •2H ₂ O	3.65g	10 min

- Add formaldehyde immediately before use.
- Make all solutions up to 250 mL with the addition of ddi water.

After staining is finished, wash gels in ddI water 3x5 min. Add glycerol (~15mL) to gels in ddI water (strengths the gels)

Make sure to take a scan and save picture in the lab notebook.